

Properties of
Zinc-Bound Water in
Horse Liver Alcohol Dehydrogenase

Bertil Oldén



Lund 1984



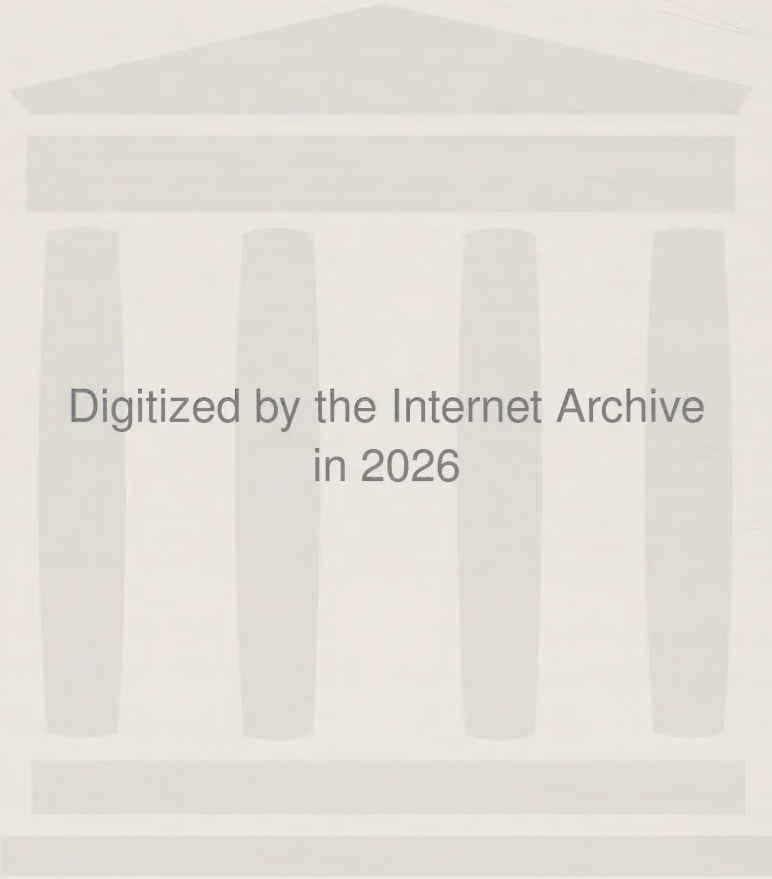
PROPERTIES OF
ZINC - BOUND WATER IN
HORSE LIVER ALCOHOL DEHYDROGENASE

by

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LUND 1984

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PUBLICATIONS

This thesis summarizes the contents of the following publications, which will be referred to by their Roman numerals:

I. Evidence that Ionization of Zinc-Bound Water Regulates the Anion-Binding Capacity of the Coenzyme-Binding Site in Liver Alcohol Dehydrogenase

Pia Andersson, Jan Kvassman, Anders Lindström, Bertil Oldén and Gösta Pettersson (1980)

Eur. J. Biochem. 108, 303 - 312.

II. Effect of NADH on the pKa of Zinc-Bound Water in Liver Alcohol Dehydrogenase

Pia Andersson, Jan Kvassman, Anders Lindström, Bertil Oldén and Gösta Pettersson (1981)

Eur. J. Biochem. 113, 425 - 433.

III. Synergism Between Coenzyme and Carboxylate Binding to Liver Alcohol Dehydrogenase

Pia Andersson, Jan Kvassman, Bertil Oldén and Gösta Pettersson (1981)

Eur. J. Biochem. 118, 119 - 123.

IV. Electrostatic Field Effects of Coenzymes on Ligand Binding to Catalytic Zinc in Liver Alcohol Dehydrogenase

Pia Andersson, Jan Kvassman, Bertil Oldén and Gösta Pettersson (1984)

Eur. J. Biochem. 138, 603 - 609.

V. Anion Binding to Liver Alcohol Dehydrogenase

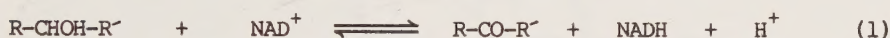
Bertil Oldén and Gösta Pettersson (1982).

Eur. J. Biochem. 125, 311 - 315.

1. INTRODUCTION

1.1 Enzyme considered

Alcohol dehydrogenases (EC 1.1.1.1) from various sources catalyse the oxidation of primary and secondary alcohols to the corresponding aldehydes or ketones (eqn. 1). They belong to the abundant and metabolically important group of enzymes which depend upon nicotinamide adenine dinucleotide coenzymes for activity. They are of interest also as representatives of the group of metallo-enzymes; in contrast to most other dehydrogenases the alcohol enzymes contain firmly protein-bound zinc.



The present thesis deals with the alcohol dehydrogenase isolated from horse liver (abbreviated LADH). This classical enzyme was first crystallized by Bonnichsen and Wassén in 1948. Shortly afterwards, Theorell and coworkers characterized fundamental kinetic and binding properties of LADH in a series of pioneering enzymological studies. Since that time, the physico-chemical and mechanistic properties of LADH have been extensively studied by a variety of experimental approaches. Determinations of the amino acid sequence of the enzyme subunit were completed by Jörnvall in 1970 (1), and have been followed up by x-ray crystallographic studies of free enzyme and a large number of enzymic complexes. The latter work, carried out by Bränden and coworkers, has provided a firm structural background for the interpretation of kinetic and spectroscopic data obtained in solution. Results reported during the last decade have led to an exceptionally detailed understanding of several fundamental features of the mechanism of action of the enzyme. The available information on the structure and function of LADH has been summarized in several recent review articles (2,3,4). It may suffice, therefore, to draw attention here to some information which is of particular importance with respect to problems considered in the present thesis.

1.2 Structure of the enzyme

The LADH molecule is a dimer consisting of two structurally identical 40000 mol wt subunits (5). Each subunit contains two firmly bound zinc ions, one of which is catalytically essential. The catalytic zinc is positioned in a hydrophobic pocket (Fig. 1) and is bound to the protein by two cysteine sulfur atoms and one histidine nitrogen atom (2). A water molecule which projects into the hydrophobic pocket completes a tetrahedral coordination to the metal (6). This water molecule is displaced by zinc-chelating reagents such as 1,10-phenantroline and imidazole (7).

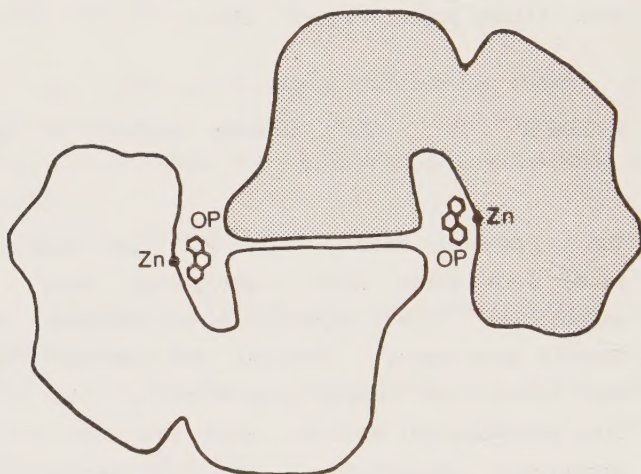


Fig. 1. Schematic diagram of a section through the dimeric LADH molecule showing the position of bound 1,10-phenantroline (OP) in relation to catalytic zinc (7).

The coenzyme binds in a specific crevice with the nicotinamide ring positioned in proximity to catalytic zinc and with the adenine moiety anchored in a hydrophobic pocket at the enzyme surface. Besides being dependent on hydrophobic interactions, coenzyme binding involves formation of a number of hydrogen bonds between polar groups of the nucleotide and side chain or main chain atoms of the enzyme. The guanidinium group of the amino acid

residue Arg-47 provides a positive charge for ionic interaction with the pyrophosphate group of coenzyme (4). The pyrophosphate-binding region of the coenzyme-binding site also functions as a binding site for anions in general (2,6).

After complex formation between enzyme and coenzyme, catalytic zinc is accessible to solution only via a barrel lined by hydrophobic protein residues (Fig. 2). This hydrophobic barrel functions as a binding site for the alcohol and aldehyde substrates (4).

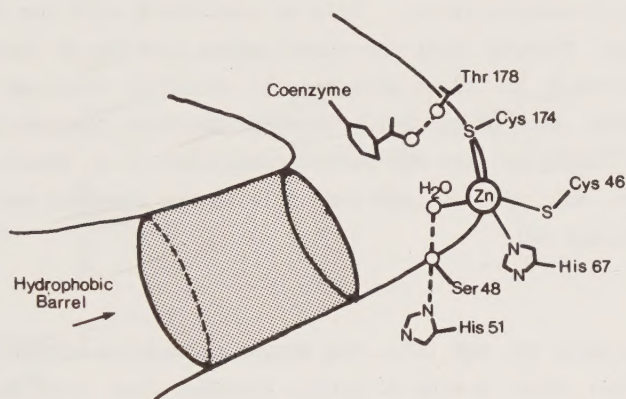


Fig. 2. Schematic diagram of the active site of LADH (8).

1.3 Mechanism of enzyme action

Kinetic studies have established that LADH operates by a ternary-complex mechanism which is usually described as effectively ordered (Scheme 1) with coenzyme binding preceding binding of the alcohol or aldehyde substrate (9).



Scheme 2. The ordered ternary complex mechanism for LADH catalysis (E=LADH, O=NAD⁺, P=alcohol, S=aldehyde, R=NADH).

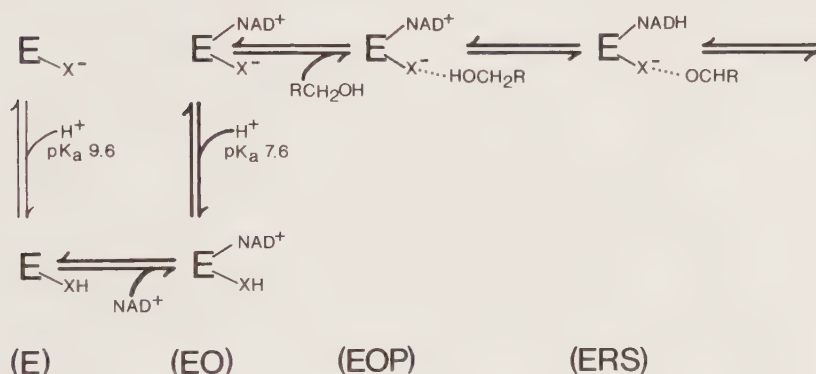
Oxidation/reduction of the coenzyme thus appears to proceed by a step of direct hydride ion transfer between coenzyme and substrate at the ternary-complex level. This is consistent with the structural information showing that the nicotinamide portion of bound coenzyme is positioned in close proximity to catalytic zinc and the substrate-binding site (Fig. 2). Evidence has been presented which indicates that catalytic zinc may participate directly in substrate binding and hence may play an important role in the chemical activation of the substrate (4).

According to eqn (1), the enzyme process of alcohol oxidation must involve also a step of proton transfer from substrate to solution. Information about the mechanism for the latter reaction step can be obtained, in principle, by examination of the pH dependence of the catalytic process. Several reaction steps in Scheme 1 are affected by pH, however, and different interpretations of the observed pH dependencies have led to different proposals as to the mechanism for the catalytic proton transfer step (2,10,11,12).

Attention was thus early drawn to the observation that NAD⁺ binding to LADH is dependent on the protonation state of an ionizing group XH which shows a pK_a of about 7.6 in the enzyme-NAD⁺ complex and a pK_a above 9 in free enzyme (10,13,14).

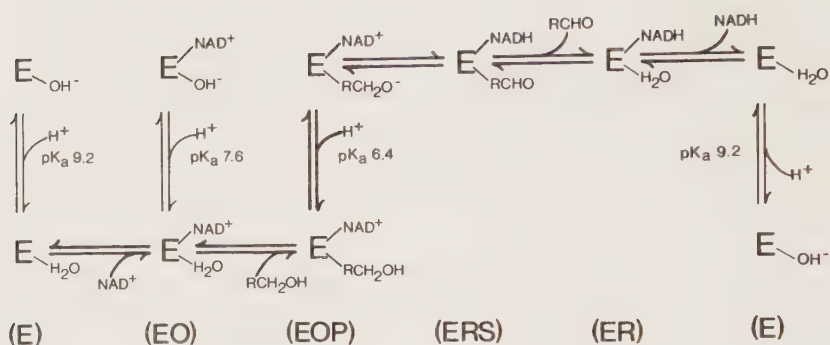
Shore et al. (1974) (10) showed that this group regulates also alcohol binding to the enzyme-NAD⁺ complex and put forward the mechanism of enzyme action indicated in Scheme 2. According to this scheme, NAD⁺

binding increases the acidity of the group XH thus triggering deprotonation of the group at the binary-complex level. The ionized group X^- then serves as a binding site for the alcohol substrate and as the immediate acceptor for proton transfer from the substrate. A modification of the mechanism in Scheme 2 was proposed by Bränden et al. (1975) who identified the group XH as the water molecule coordinated to catalytic zinc, and who suggested that the zinc-bound hydroxyl ion functions as a catalyst for the formation of a zinc-bound alcoholate ion (2). Dworschack et al. (1977) have proposed a similar reaction mechanism according to which the zinc-bound hydroxyl ion functions as a base catalyst of hydride transfer from a penta-coordinated zinc-bound alcohol to NAD^+ (11).



Scheme 2. Mechanism proposed by Shore et al. (1975)

In a recent series of investigations, Kvassman and Pettersson (15,16,17) examined the effects of pH on all individual reaction steps in Scheme 1. This work led them to propose (12) the mechanism shown in Scheme 3. Kvassman and Pettersson favoured the idea that observed effects of pH on coenzyme binding reflect the ionization of a single enzymic group, probably zinc-bound water. In contrast to other investigators, however, they do not attribute any essential catalytic role to this group - the pKa 9.2 and 7.6 ionization steps in Scheme 3 are side reactions in the catalytic cycle. Instead, the obligatory proton transfer step is represented by an alcohol/alcoholate ion equilibration (pKa 6.4) in the ternary complex.



Scheme 3. Mechanism proposed by Kvassman and Pettersson (1980).

1.4 Aims of the present work

It seems obvious from the above mechanistic proposals that our understanding of the binding properties and catalytic activity of LADH is critically dependent on knowledge about the protonation equilibria which affect the catalytic reaction and the identity of the corresponding ionizing groups. The mechanism proposed by Kvassman and Pettersson (Scheme 3) is of particular interest in this respect, since it accounts for all observed effects of pH and attribute them to a common chemical event; proton equilibria in Scheme 3 were assumed to reflect deprotonation of a zinc-bound hydroxyl group either from water or from the alcohol substrate. Studies summarized in the present thesis, therefore, have focused on the ionization properties of zinc-bound water in LADH and on the effect of ionization of zinc-bound water on the binding properties of the enzyme.

2. THE IDENTITY OF THE IONIZING GROUP AFFECTING COENZYME BINDING

Coenzyme binding to LADH is affected by two protonation equilibria within the pH range 6 - 10 (Scheme 3). The ionization involving free enzyme (pKa 9.2) regulates the association of both coenzyme forms. The second ionization step (pKa 7.6) controls the rate of NAD^+ dissociation from the enzyme-coenzyme complex, while there is no corresponding effect of pH on the rate of NADH desorption. This means that the equilibrium constant for NADH binding remains essentially pH independent over the pH range 6 - 9 where the equilibrium binding of NAD^+ is strongly affected by pH (Fig. 3).

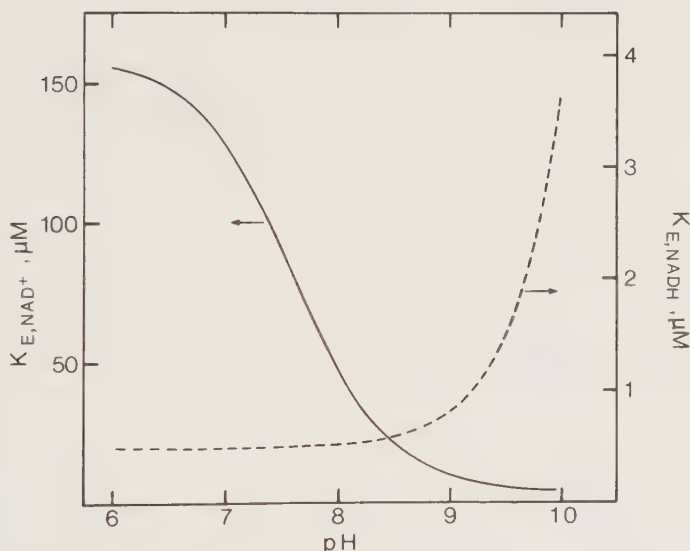


Fig. 3. Effect of pH on the equilibrium constants for NADH (-----) and NAD^+ (————) dissociation from LADH.

Theorell et al. (1961) early drew attention to the fact that the differential effects of pH on the binding of oxidized and reduced coenzyme must reflect differential interactions of NADH and NAD^+ with

an ionizing enzymic group (14). Their observation that the pH dependence of NAD^+ binding is abolished on complex formation between enzyme and the zinc-chelating reagent imidazole led them to identify the ionizing group as a water molecule bound to the catalytic zinc ion. Zinc-bound water was assumed to show a lower pK_a in the enzyme- NAD^+ complex than in free enzyme (cf Scheme 3) due to electrostatic stabilization of the zinc-bound hydroxyl ion by the positively charged nicotinamide ring of NAD^+ .

When the present work was initiated, strong evidence had accumulated which supported the proposal of Theorell et al. that zinc-bound water exhibits an electrostatically perturbed pK_a in the enzyme- NAD^+ complex (pK_a 7.6 in Scheme 3). Crystallographic data presented by Bränden and coworkers confirmed that a water molecule is coordinated to the catalytic zinc ion (2) and demonstrated that this water molecule is the only ionizing enzymic group which is structurally affected (displaced) by imidazole binding (7). The nicotinamide ring of enzyme-bound NAD^+ was found to be positioned in close proximity to zinc-bound water and would thus be expected to interact strongly with the zinc-bound hydroxyl ion. This made it easy to understand why NAD^+ (but not NADH) perturbs the pK_a of the group, and why ionization of the group results in a decreased rate of NAD^+ dissociation from the enzyme. The pK_a -7.6-dependence of alcohol binding to the enzyme- NAD^+ complex (10), similarly, can be readily explained in terms of ionization of zinc-bound water (16).

On the other hand, there was reason to question (13,16) the proposal of Theorell et al. (14) that the pH dependence of NAD^+ binding derives from a single ionizing group, i.e. that zinc-bound water accounts also for the pK_a -9.2-dependence of coenzyme binding. To be consistent with data in Fig. 3, the latter proposal would require that the pK_a of zinc-bound water increases to a value above 10 on NADH binding to the enzyme (13,16). No thermodynamic explanation for such a NADH -induced pK_a perturbation had been put forward, nor was it easy to understand why ionization of zinc-bound water should affect the rates of NADH and NAD^+ association to free enzyme. Furthermore, the assignment of a pK_a value of 9.2 to zinc-bound water in free enzyme seemed difficult to reconcile with the fact that the catalytic zinc ion is coordinated

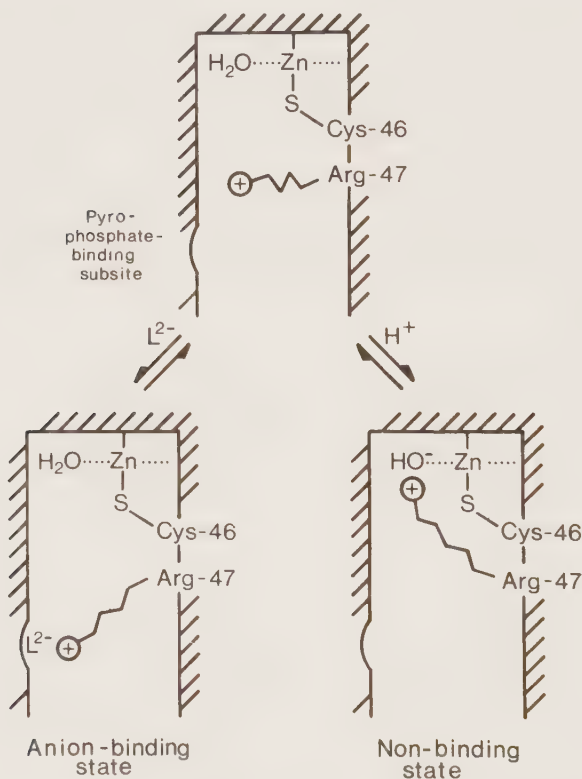
to two negatively charged cysteinyl residues (2). It appeared more reasonable to assume that zinc-bound water ionizes with a pKa above 10 in both free enzyme and the enzyme-NADH complex, which would imply that the pKa-9.2-dependence of coenzyme binding derives from a second ionizing group. In fact, no evidence was available which related the pKa-9.2-dependence of coenzyme binding to catalytic zinc or to ionization of zinc-bound water. On the contrary, data reported for the binding of ligands such as platinum cyanide, ADP-ribose and AMP (16,18) provided clear evidence that effects of ionization of the pKa-9.2-group are related to the pyrophosphate-binding subsite, which is located at some distance from catalytic zinc.

For these reasons, we considered it important to obtain more conclusive information about the pKa of zinc-bound water in free enzyme. Ionization of zinc-bound water can be anticipated to strongly affect the binding properties of catalytic zinc. Consequently, we examined the effect of pH on the binding of ligands such as 1,10-phenantroline, bipyridine and imidazole which are known to combine to catalytic zinc on complex formation with the enzyme (7,19) (Paper I).

Results described in Paper I show that the pKa 9.2 dependence of NADH binding is abolished on complex formation between enzyme and imidazole as would be expected if the pKa 9.2 dependence derives from ionization of zinc-bound water. The observation that complex formation between enzyme and bipyridine exhibits a pKa 9.2 dependence analogous to that of coenzyme binding provides more direct evidence for ionization of zinc-bound water with a pKa of 9.2 in free enzyme. Paper I also presents evidence showing that the pKa-9.2-dependence of bipyridine binding is abolished by coenzyme-competitive ligands such as platinum cyanide and ADP-ribose. The latter observation is of particular interest as it demonstrates that there is a mechanistic interrelationship between the ionizing group, ligand binding at catalytic zinc and complex formation at the pyrophosphate-binding subsite of the coenzyme-binding site.

We concluded from these results that zinc-bound water most likely shows a pKa of 9.2 in free enzyme, and that the ionization state of

this group controls not only ligand binding to the catalytic zinc ion but also the binding of coenzymes and other anionic ligands which utilize the pyrophosphate-binding subsite of the coenzyme-binding site. Ionization of zinc-bound water can be anticipated to trigger (more or less extensive) conformation changes of the enzyme. We suggested that such conformational adjustments lead to salt-bridge formation between the zinc-bound hydroxyl ion and the guanidinium group of Arg-47 (Scheme 4), which is the main cationic constituent of the pyrophosphate-binding subsite. This made it possible to explain why ionization of zinc-bound water prevents complex formation at the latter site, why such ionization affects the coenzyme association



Scheme 4. Competitive salt bridge formation proposed to account for the competitive interrelationship between ionization of zinc-bound water and complex formation with anionic ligands at the pyrophosphate-binding subsite of the coenzyme-binding site.

rates, why zinc-bound water shows an exceptionally low pKa in free enzyme, and why the pKa of zinc-bound water increases on the binding of NADH and other anionic ligands which utilize the pyrophosphate-binding subsite. Paper I thus provided a reasonable explanation for all of the observations which had seemed difficult to reconcile with the idea that the pH dependence of coenzyme binding derives only from ionization of zinc-bound water.

3. MECHANISM FOR COMPLEX FORMATION AT THE CATALYTIC ZINC ION

The results in Paper I give some information also on the mechanism for ligand binding at the catalytic zinc ion. Previously reported data for chelation of bidentate ligands to catalytic zinc had been interpreted in terms of a two-step binding mechanism (20,21). The association of bidentate ligands exhibits a limiting rate of about 250 s^{-1} which was proposed to reflect the dissociation of zinc-bound water. No limitation of the association rate of monodentate ligands could be detected, which led Frolich et al. (1978) to propose that substrates combine to catalytic zinc without any displacement of zinc-bound water i.e. that a penta-coordinate metal complex forms upon substrate binding to the enzyme (21).

The pH dependence data in Paper I, however, establish that zinc-bound water affects only the first of the two reaction steps by which bipyridine is bound. This indicates that zinc-bound water is displaced in the primary step of ligand association, probably through a monodentate inner-sphere coordination of bipyridine to zinc. The subsequent rate-limiting step can be envisaged to reflect bidentate chelate formation through penta-coordination of the metal. Interpreted along these lines, the absence of a limiting association rate of monodentate ligands indicates a binding mechanism which does not involve any change in the coordination number of catalytic zinc, i.e. provides evidence that substrates combine to the catalytic zinc ion with displacement of zinc-bound water.

4. THE pKa OF ZINC-BOUND WATER IN THE BINARY ENZYME-NADH COMPLEX

Results in Paper I provided strong evidence favouring the idea (14) that coenzyme binding to LADH is regulated by a single ionizing group (zinc-bound water), the pKa of which is perturbed from 9.2 in free enzyme to 7.6 in the enzyme-NAD⁺ complex and to a value above 10 in the enzyme-NADH complex. It became of interest, therefore, to obtain more precise information about the latter pKa value by examination of the effect of pH on coenzyme binding above pH 10. Due to the restricted stability of enzyme and coenzymes at such high pH, pH jump techniques had to be developed and applied for these measurements. By such methods, equilibrium binding studies could be performed over the pH range 10 - 12 (Paper II).

The results showed that NADH binding is affected by two protonation equilibria between pH 10 and 12. The equilibrium with a pKa value of 10.4 exhibits a functional dependence which implies that this pKa reflects the ionization state of a group distinct from zinc-bound water. This conclusion was corroborated by the observation that the binding of NAD⁺ exhibits a pKa 10.4 dependence analogous to that of NADH binding. The pKa 11.2 dependence of NADH binding, however, might derive from ionization of zinc-bound water in the enzyme-NADH complex. If so, then aldehyde binding to the enzyme-NADH complex should exhibit a pKa 11.2 dependence as well. Our observation that binding of trans-N,N-dimethylaminocinnamaldehyde requires the protonated form of a pKa 11.2 group, therefore, provides confirmatory evidence that zinc-bound water ionizes with a pKa value of 11.2 in the binary enzyme-NADH complex.

We suggested in Paper I that ionization of zinc-bound water is facilitated by the stabilizing effect of the positive guanidinium group of Arg-47. This explains why zinc-bound water exhibits a pKa value as low as 9.2 in free enzyme despite the fact that it is coordinated to a zinc ion which is bound by two negatively charged cysteine ligands. According to this view, zinc-bound water should exhibit a more typical pKa in the binary enzyme-NADH complex where

the guanidinium group is engaged in salt-bridge formation at the pyrophosphate-binding subsite. Indeed, the pKa value of 11.2 assigned to zinc-bound water in the latter complex is in excellent agreement with the value expected from the reported pKa of metal-bound water in other zinc-containing enzymes (Table 1) and in model zinc complexes (22).

Table 1. Correlation between inner-sphere ligand field and the pKa of zinc-bound water in enzymes containing a four-coordinate active site zinc ion.

Enzyme	Protein ligand	Total ligand charge	pKa	Ref.
Carbonic anhydrase	His-93 His-95 His-117	0	7.0	(23)
Carboxypeptidase	His-142 His-146 Glu-166	-1	8.9	(24)
LADH	His-67 Cys-46 Cys-174	-2	11.2	Paper II

5. ELECTROSTATIC FIELD EFFECTS OF COENZYMES ON THE pKa OF ZINC-BOUND WATER

Theorell et al. (14) attributed the pH dependence of NAD⁺ binding to electrostatic field effects of the positively charged nicotinamide ring on the ionization properties of zinc-bound water. The discussion in Paper I shows that the pH dependence of NADH binding can be explained in similar terms. The postulated perturbation of the pKa of zinc-bound water from 9.2 in free enzyme to 11.2 in the enzyme-NADH complex can thus be thermodynamically attributed to the destabilizing effect of the negatively charged coenzyme pyrophosphate group on a zinc-bound hydroxyl ion. Arg-47 only serves as a (likely) mediator of this interaction. The same destabilizing interaction must obviously

be at hand in the enzyme-NAD⁺ complex, but is counteracted by the stabilizing effect of the charged nicotinamide ring of the oxidized coenzyme. Crystallographic data show that the positive charge on enzyme-bound NAD⁺ is located in much closer proximity to the catalytic zinc ion than is the negatively charged pyrophosphate group (4). The binding of NAD⁺, therefore, should result in net stabilization of the zinc-bound hydroxyl ion. This accounts for the decrease in pK_a of zinc-bound water from 9.2 in free enzyme to a value of 7.6 in the enzyme-NAD⁺ complex.

Thus, the differences in magnitude of the pK_a values of zinc-bound water in free enzyme and the enzyme-coenzyme complexes can be envisaged to reflect electrostatic interactions between coenzyme and the zinc-bound hydroxyl ion. This means that anion binding to the active-site zinc ion should be generally subjected to coenzyme-induced electrostatic field effects of similar strength. In Paper III and IV we test the significance of such effects by examining the synergism between coenzyme binding and the binding of negatively charged ligands which are likely to combine to the active-site zinc ion on complex formation with the enzyme.

The results in Paper III demonstrate that complex formation between coenzyme and carboxylates (benzoate and decanoate) requires the protonated form of an ionizing group with a pK_a value of 9.2, consistent with the idea that carboxylates bind to the active-site zinc ion of the enzyme (7) with displacement of zinc-bound water. Results in Paper IV establish that cyanide is bound as CN⁻ and indicate that CN⁻ binding is similarly dependent on the ionizing state of the pK_a 9.2 group (zinc-bound water). The binding of CN⁻ was found to be strictly competitive with the ligation of bipyridine to catalytic zinc. Furthermore, ternary enzyme-CN⁻-coenzyme complexes form with both NAD⁺ and NADH, which provides clear evidence that CN⁻ interacts with a site distinct from the pyrophosphate-binding subsite. Considering also the exceptional metal-coordinative properties of the ligand, there seems to be no doubt that CN⁻ combines to catalytic zinc ion on complex formation with the enzyme.

The affinity of the enzyme for CN^- decreases (increases) on the binding of NADH (NAD^+). Since zinc-bound cyanide is unlikely to interfere sterically with coenzyme binding (2), the observed synergism can be assumed to derive almost exclusively from electrostatic interactions. The cooperativity factors determined for coenzyme and CN^- binding agree well in magnitude with those obtained for the synergism between coenzymes and decanoate binding, confirming that the effects observed are attributable to charge interactions. The interaction between enzyme and decanoate is weakened not only on complex formation with NADH, but also on binding of the coenzyme-competitive inhibitors ADP-ribose and platinum cyanide. This indicates that the destabilizing effect of NADH on complex formation at the catalytic zinc ion derives mainly from the negatively charged pyrophosphate group of the coenzyme. The opposite effect of NAD^+ can be envisaged to derive from its positively charged nicotinamide ring.

Table 2 compares the coenzyme-induced effects on the binding of anionic ligands to catalytic zinc with the effects of coenzymes on the pK_a values attributed to deprotonation of zinc-bound water. It can be inferred from these data that there is a striking analogy and quantitative agreement between the coenzyme-induced effects on the binding of CN^- and decanoate on one hand, and proton binding, on the other. Results summarized in Table 2 are in obvious consistence with, and lend strong support to, the idea that the pH dependence of coenzyme binding derives from electrostatic field effects of the coenzymes on the ionization of zinc-bound water.

Table 2. Effect of coenzymes on pK_a values attributed to deprotonation of zinc-bound water (16,II) and pK values for anion dissociation from the catalytic zinc ion (14,III,IV).

Species	H^+		$\text{C}_9\text{H}_{19}\text{COO}^-$	CN^-
	pK_a	ΔpK_a	ΔpK	ΔpK
Enzyme	9.2			
Enzyme- NAD^+	7.6	-1.6	+1.6	+1.7
Enzyme-NADH	11.2	+2.0	-1.7	-1.7

6. THE INTERACTION OF ANIONS WITH THE PYROPHOSPHATE BINDING SUBSITE

During the course of this work, Dahl et al. (25) reported that phosphate combines to the pyrophosphate-binding subsite of LADH with an apparent dissociation constant as low as 20 mM at pH 7. This report was alarming in the sense that it drew attention to the lack of detailed knowledge about the affinity of the pyrophosphate-binding subsite for anions in general, and anionic species of buffer solutions in particular. As was pointed out in Paper I, all anions which combine to the pyrophosphate-binding subsite can be anticipated to perturb the pKa of the ionizing group identified as zinc-bound water. Effects of buffer anion binding had been considered as insignificant, however, in the binding and pH dependence studies performed by us and others. To estimate the magnitude of previously unaccounted effects of buffer anion binding to the enzyme, we carried out a systematic study of anion binding by examining the effects of various neutral salts and buffer systems on the transient-state kinetics of complex formation between enzyme and NADH (Paper V).

The binding of representative anions was shown not to be competitive with the binding of bipyridine to the catalytic zinc ion. This was taken as evidence that anion dissociation constants calculated from the observed competitive effects of anions on NADH binding reflect interactions with the pyrophosphate-binding subsite. Results thus obtained (Table 3) indicate that the binding of neutral salt anions to the pyrophosphate-binding subsite conforms essentially to the well-known lyotropic (Hofmeister) series of affinity order (26,27). The affinity of the enzyme for sulphate deviates from this series which may indicate that the pyrophosphate-binding subsite exhibits an extraordinary specificity for this anion.

Anionic components of phosphate, carbonate and pyrophosphate buffer solutions were found to show a low affinity for the pyrophosphate-binding subsite. Coenzyme-competitive phosphate binding, for example, occurred with an apparent dissociation constant of about 90 mM at pH 7 (and was shown to be attributable to the H_2PO_4^- species). The tighter

Table 3. Dissociation constants for anion binding to the pyrophosphate-binding subsite of the coenzyme-binding site.

Anion	K
	mM
F ⁻	>200
CH ₃ COO ⁻	60
Cl ⁻	42
HCOO ⁻	37
Br ⁻	19
SO ₄ ²⁻	8.8
NO ₃ ⁻	8.4
I ⁻	5.3
ICH ₂ COO ⁻	4.8
ClO ₄ ⁻	3.5
SCN ⁻	1.4
Pt(CN) ₄ ²⁻	0.04

binding of phosphate indicated by the Cys-46 alkylation experiments of Dahl et al. (1982) might reflect buffer effects which are not coenzyme-competitive. We concluded from the results in Paper V that the pH dependence data for coenzyme binding, and hence the pKa values for zinc-bound water, reported from our laboratory cannot be much affected by unaccounted effects of coenzyme-competitive buffer anion binding.

7. THE INTERACTION OF ANIONS WITH THE CATALYTIC ZINC ION

Some additional information on the anion-binding properties of LADH was gathered from the results in Paper IV. As indicated by Table 2, the binding of anions to the active-site zinc ion appears to be about two orders of magnitude tighter in the presence of enzyme bound NAD⁺ than in the absence of coenzyme. Consequently, complex formation between enzyme and NAD⁺ might render anion binding to catalytic zinc detectably tight also with ligands which combine preferentially

to the pyrophosphate-binding subsite in their interaction with free enzyme. Kinetic data for the binding of SCN^- and acetate are interpreted in terms of such ligation to the metal in the enzyme- NAD^+ -anion complex. It follows from these data that interactions with the catalytic zinc ion, thought of minor importance in the binary enzyme-anion complex, might be of significance for the inhibitory effects of SCN^- and acetate on the catalytic activity of the enzyme. The failure to demonstrate ligation to zinc with other anions, indicates that anions in general interact at least 200 times more strongly with the pyrophosphate-binding subsite than with catalytic zinc in free enzyme. This corroborates previous conclusions (6,28,29,30) that the pyrophosphate-binding subsite functions as the main binding site for anions in general.

8. GENERAL DISCUSSION

The origin and mechanistic significance of the pH dependence of coenzyme binding to LADH has been the subject of much debate. Theorell et al. (14) were the first to relate the observed effects of pH on NAD^+ binding to zinc-bound water, as well as to electrostatic interactions of the positive nicotinamide ring charge of NAD^+ on the ionizing pKa-7.6-group in the enzyme- NAD^+ complex. Kvassman and Pettersson (12) argued from the available structural information on the enzyme and its ionizing groups that the ring charge of oxidized coenzyme is the only structural difference between NADH and NAD^+ which could account for any pronounced differences on their pH dependence. Hence they concluded that the pKa-7.6-group must be an electrostatically perturbed one, and pointed out that zinc-bound water was the most likely candidate in view of the observed magnitude of the corresponding pKa perturbation.

Results summarized in the present thesis make it possible to develop the above arguments in full detail (Paper IV). The pH dependence of

NADH and NAD^+ binding reflects the synergism between coenzyme and proton binding to the enzyme, and the NAD^+ ring charge must favour deprotonation of the enzyme irrespective of the number or nature of the ionizing groups which function as proton acceptors. Consequently, effects of pH on the relative magnitude of dissociation equilibrium constants for NAD^+ ($K_{\text{E,NAD}^+}$) and NADH ($K_{\text{E,NADH}}$) must include contributions from all ionizing groups which interact strongly enough with the NAD^+ ring charge. Results in Paper II are of particular interest in this context. They extend our knowledge about the differential effects of pH on NADH and NAD^+ binding from the pH range 6 - 10 (Fig. 3) to the range 6 - 12, providing the information on the pH dependence of $K_{\text{E,NAD}^+}/K_{\text{E,NADH}}$ illustrated by the data in Fig. 4.

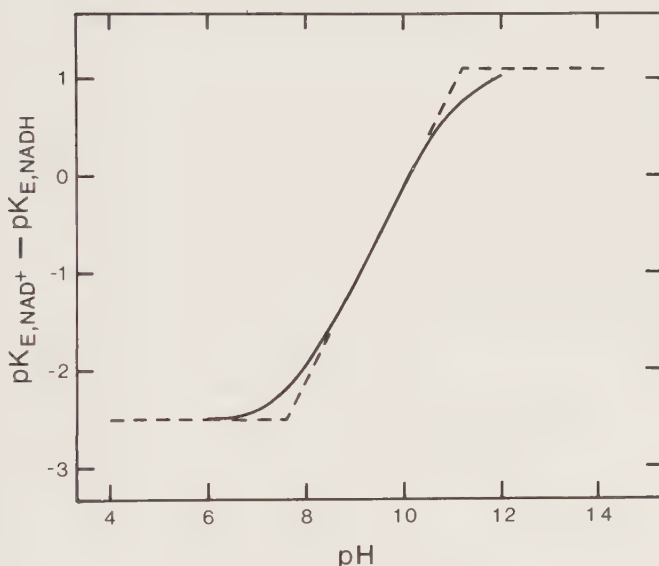


Fig. 4. Electrostatic field effect of the positively charged nicotinamide ring of NAD^+ on proton binding to the enzyme.

As discussed in Paper IV several clarifying inferences can be drawn from the latter data. Firstly, there is only one ionizing enzymic group which interacts strongly enough with the NAD^+ ring charge to cause any significant differences in the pH dependence of NADH and

NAD⁺ binding between pH 6 and 12. Other ionizing enzyme groups either interact weakly with the NAD⁺ ring charge, or show pKa values falling (on the same side) outside the range 6 - 12 in the enzyme-NAD⁺ and enzyme-NADH complex. Secondly, the pH effect in Fig. 4 is caused by the group which accounts for the pKa-7.6-dependence of NAD⁺ binding and the pKa-11.2-dependence of NADH binding; attribution of these pH dependencies to distinct ionizing groups is not compatible with the experimental results. Finally, the interaction of the NAD⁺ ring charge with this ionizing group is of exceptional strength, leading to a pKa perturbation of 3.6 units. This means that the ionizing group must be positioned in close proximity to the nicotinamide ring of enzyme-bound NAD⁺. Results in Paper II, therefore, reinforce the conclusion by Kvassman and Pettersson (12): the ionizing properties of the pKa 7.6/11.2 group place such restrictions on its nature and location in the enzyme that zinc-bound water appears to be the only reasonable alternative available.

Results in Paper III and Paper IV provide direct experimental estimates of the synergistic effects of NADH and NAD⁺ on anion binding to catalytic zinc (Table 2), estimates which can be taken to be representative also for the synergistic effects of the coenzymes on hydroxyl ion binding to catalytic zinc, i. e. on ionization of zinc bound water. Data in Table 2 establish that the electrostatic field effect ($\Delta pK_a = 3.3 - 3.4$) of the NAD⁺ ring charge on anion binding to catalytic zinc is precisely (within experimental precision) of the magnitude required to account for the observed pKa perturbation by 3.6 of the ionizing pKa 7.6/11.2 group in terms of ionization of zinc-bound water. Furthermore, it follows from anion binding data in Table 2 that zinc-bound water must be assumed to undergo such a drastic pKa perturbation by the NAD⁺ ring charge irrespective of the identity of the pKa 7.6/11.2 group. In other words, if the drastic pH effect illustrated in Fig. 4 is assumed not to derive from zinc-bound water, then the corresponding (and equally drastic) effect attributable to zinc-bound water must fall entirely below pH 6 or entirely above pH 12. As discussed in Paper IV, the latter two alternatives are ruled out by present knowledge about the coordination chemistry of zinc in general and the catalytic zinc ion of LADH in particular. Attribution of the pH effect in Fig. 4 to an ionizing group distinct from zinc-bound water would not only make it difficult to understand the ioni-

zing properties of the group which is assumed to cause the pH effect, it would also require unreasonable assumptions about the ionization properties of zinc-bound water to explain the absence of detectable effects of the latter group.

On such grounds, results in Paper III and IV can be taken to provide conclusive evidence identifying the pKa-7.6/11.2-group as zinc-bound water. It then follows from the same results that zinc-bound water must be assumed to account also for the pKa-9.2-dependence of coenzyme binding to free enzyme, there is no reasonable alternative interpretation of the qualitative and quantitative correlations documented by data in Table 2. Development of the thermodynamic arguments put forward by Theorell et al. (14) and Kvassman and Pettersson (16), therefore, leads to an unambiguous attribution of the pKa 7.6/9.2/11.2-dependencies of coenzyme binding to zinc-bound water, confirmed also by the evidence presented in Paper I.

Bränden et al. (2) have presented crystallographic evidence showing that zinc-bound water forms part of an extended hydrogen-bonded structure which may involve the side-chains of Ser-48 and His-51 as well as polar groups on the coenzymes. The detailed bonding interactions of the coenzymes with LADH, and their dependence on the ionization state of zinc-bound water, thus appears to be a matter of considerable complexity. This calls for some caution as concerns the detailed interpretation of results presented in this thesis. Proposals in Paper I regarding effects of pH on the kinetics of coenzyme binding, for example, will remain tentative as long as we have no information on the rate-limiting steps of the processes of coenzyme association and dissociation, or on the enzyme conformational changes which may be associated with the ionization of zinc-bound water.

9. CONCLUDING REMARKS

Results summarized in this thesis confirm that coenzyme binding to liver alcohol dehydrogenase is dependent on an ionizing group with a pK_a value of 9.2 in free enzyme, decreasing to 7.6 in the enzyme-NAD⁺ complex and increasing to a value above 10 in the enzyme-NADH complex. The results are fully consistent with, and would seem to provide conclusive evidence in support of, the idea that these effects of pH on coenzyme binding reflect the ionization state of the water molecule which is coordinated by the active-site zinc ion. This eliminates also previous ambiguities as to the interpretation of observed effects (10,12,17,31,32,I,II) or lack of effects (17,33,34,III) of the ionizing group on the binding of substrates and certain substrate analogues. The latter observations can be readily rationalized in terms of complex formation leading to, or not leading to, inner-sphere coordination of the substrate and substrate analogue to catalytic zinc. Reported pH dependence data for anion binding to the enzyme (18,35,IV), similarly, can be well understood in view of the evidence now presented for electrostatic field effects of positively and negatively charged coenzyme groups on the ionizing properties of zinc-bound water.

The present thesis, therefore, corroborates all major postulates made about the coenzyme and substrate binding properties of LADH in the mechanistic proposal of Kvassman and Pettersson (Scheme 3) (12). The evidence demonstrating that zinc-bound water and inhibiting anions are subjected to strong electrostatic field effects of the coenzymes lead to the inference that such effects must be at hand also in the productive enzyme-coenzyme-substrate complexes. This lends credence also to the proposal in Scheme 3 that NAD⁺-induced ionization of zinc-bound alcohol substrates represents a catalytically crucial reaction step in the mechanism of action of LADH.

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friends, "here and there", for encouragement

AnnaKarin, for loyalty and sacrifice

Mathias and Maja, my children. I dedicate this work to them.

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Evidence that Ionization of Zinc-Bound Water Regulates the Anion-Binding Capacity of the Coenzyme-Binding Site in Liver Alcohol Dehydrogenase

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1. The mechanistic and structural origin of the pK_a 9.2 dependence of coenzyme association and anion binding to liver alcohol dehydrogenase has been investigated by titrimetric and spectrophotometric binding studies involving ligands (imidazole, 1,10-phenanthroline and 2,2'-bipyridine) which combine to the catalytic zinc ion of the enzyme subunit with displacement of zinc-bound water.

2. Imidazole abolishes the pK_a 9.2 dependence of NADH binding to the enzyme. The pH dependence of ADP-ribose and $Pt(CN)_4^{2-}$ binding is similarly abolished by imidazole, as well as by 1,10-phenanthroline and 2,2'-bipyridine. It is concluded from these results that the pK_a 9.2 dependence of coenzyme and anion binding most likely derives from ionization of zinc-bound water.

3. Evidence is presented showing that the protonation state of the pK_a 9.2 group also regulates ligand binding to the catalytic zinc ion, which lends strong support to the conclusion that this ionizing group can be identified as zinc-bound water. The pK_a 9.2 dependence of bipyridine binding is abolished by ADP-ribose and $Pt(CN)_4^{2-}$, indicating that complex formation at the anion-binding subsite of the coenzyme-binding site perturbs the pK_a of zinc-bound water to a value above 10.

4. The cooperative interrelationship between ionization of zinc-bound water and complex formation at the anion-binding subsite is proposed to be attributable to a competition between the zinc-bound hydroxyl ion and external anionic ligands for salt bridge formation with the guanidinium group of Arg-47. This explains why ionization of zinc-bound water affects the anion-binding capacity of the enzyme and why complex formation at the anion-binding subsite affects the pK_a of zinc-bound water.

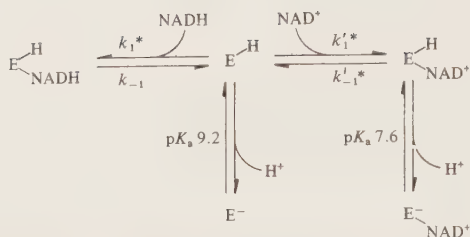
5. The kinetics of complex formation with bipyridine are consistent with a two-step binding mechanism in which a pK_a 9.2 dependent rapid pre-equilibration between enzyme and ligand is followed by a pH independent rate-limiting formation of the chromophoric binary complex. Desorption of zinc-bound water, therefore, is likely to take place in the primary association step rather than in the subsequent rate-limiting step. This renders the possibility less likely that productive ternary complexes formed during catalysis may contain a zinc-bound penta-coordinate water molecule.

In a recent report from our laboratory it was shown that the proton dissociation mechanism in Scheme 1 accounts for the main effects of pH on coenzyme binding to liver alcohol dehydrogenase over the pH range 6–10 [1]. The ionization step involving free enzyme (pK_a 9.2) was found not only to control the rates of NADH and NAD^+ association to the enzyme, but also to regulate the binding of coenzyme-competitive anions such as $Pt(CN)_4^{2-}$ and the coenzyme fragments ADP-ribose and AMP. Since there is no

similar effect of pH on binary-complex formation with adenosine [2], it was concluded that the pK_a 9.2 dependence of coenzyme association reflects ionization of a functional group which affects the anion-binding capacity of the coenzyme-binding site. This would be consistent with the report that binding of anions such as iodoacetate, chloride, and formate requires protonation of an enzymic group with a pK_a close to 9 [3].

The second ionization step in Scheme 1 (pK_a 7.6) controls the rate of NAD^+ dissociation from the enzyme · NAD^+ complex. The fact that there is no corresponding effect of pH on the rate of NADH desorption provides direct evidence that the pK_a 7.6

Enzyme. Liver alcohol dehydrogenase or alcohol : NAD^+ oxidoreductase (EC 1.1.1.1).



Scheme 1. Proton dissociation steps affecting coenzyme binding to liver alcohol dehydrogenase

step involves ionization of an electrostatically perturbed group which exhibits a pK_a value above 10 in the enzyme $\cdot$ NADH complex [1,4]. The observation that imidazole abolishes the pronounced pH dependence of NAD^+ binding led Theorell et al. to identify this ionizing group as a water molecule bound to the zinc ion at the active center of the enzyme [5]. The crystallographic results of Brändén and coworkers confirm that a water molecule is actually coordinated to the catalytic zinc ion [6] and establish that this water molecule is the only enzymic group which is affected by imidazole binding [7]. Furthermore, no other ionizing group on the enzyme appears to be located at a sufficiently short distance from the nicotinamide ring of enzyme-bound NAD^+ to undergo a large pK_a shift due to electrostatic interaction with the coenzyme [1,6]. For these reasons, there seems to be little doubt that the pK_a 7.6 dependence of NAD^+ dissociation derives from ionization of zinc-bound water.

The binding data of Theorell et al. [5] were obtained over the pH range 6–9 and provide no reliable information about the effect of imidazole on the pK_a 9.2 dependence of coenzyme association. Nevertheless, the idea is widely held that the pH dependence of NAD^+ binding is attributable to a single ionizing group [6], i.e. that both proton dissociation steps in Scheme 1 reflect ionization of zinc-bound water. It should be noted, however, that imidazole has been reported not to abolish the pH dependence of NADH binding [8]. This might suggest that the pK_a 9.2 dependence of coenzyme association derives from an ionizing group distinct from zinc-bound water, a possibility which cannot be excluded from available kinetic data [1].

We have now attempted to obtain more conclusive information about the identity of the pK_a 9.2 group through binding studies involving ligands which combine to the catalytic zinc ion with displacement of zinc-bound water. The results reported in the present paper strongly suggest that the pK_a 9.2 dependence of coenzyme and anion binding does derive from zinc-bound water. Evidence is presented indicating that

the pK_a of zinc-bound water is affected by a specific change in enzyme conformation which can be directly related to the anion-binding capacity of the coenzyme-binding site. This could explain why complex formation at the anion-binding subsite is dependent on the ionization state of zinc-bound water and why the pK_a of this group is perturbed to a value above 10 on NADH binding.

EXPERIMENTAL PROCEDURE

Materials

Crystalline horse liver alcohol dehydrogenase from Boehringer (Mannheim) was further purified as described by Bernhard et al. [9]. When required, unbuffered enzyme solutions were prepared using 33 mM sodium sulphate solution for elution of the enzyme in the column chromatographic purification step. Enzyme concentrations were determined fluorimetrically by titration with NADH in the presence of isobutyramide [10] and are reported throughout as active-site concentrations.

The coenzymes NAD^+ and NADH (grade III quality) were purchased from Sigma Chemical Corp. (St Louis, Missouri) and purified as described by Gurr et al. [11]. Other reagents were of highest available quality and used without further purification. ADP-ribose and imidazole (grade III) were obtained from Sigma Chemical Corp., $K_2Pt(CN)_4$ and 2,2'-bipyridine from Fluka AG (Buchs).

All experiments reported in the present paper were carried out at 25 °C in solutions of 0.1 M ionic strength. Titrimetric measurements were performed in 33 mM sodium sulphate, and other experiments in phosphate (pH 7–9) or bicarbonate (pH 9–10) buffer.

Titrimetric Experiments

Measurements of proton uptake resulting from the binding of the anionic ligands NADH, ADP-ribose, or $Pt(CN)_4^{2-}$ to free enzyme or the binary enzymic complexes formed with zinc-coordinating inhibitors imidazole, 1,10-phenanthroline, or 2,2'-bipyridine were performed by titrimetric techniques as detailed previously [1]. Binary enzyme $\cdot$ inhibitor complexes were prepared by preincubation of the enzyme with 40 mM imidazole, 2 mM 1,10-phenanthroline, or 20 mM 2,2'-bipyridine, sufficient to obtain at least 93% saturation of the enzyme according to binding data reported previously [8,12] or in the present paper. Anionic ligands were added in concentrations high enough to give an equilibrium solution containing more than 90% of the enzyme in the form of a ternary enzyme inhibitor $\cdot$ anion complex {0.4 mM NADH [5,8], 4 mM ADP-ribose [12], or 10 mM $Pt(CN)_4^{2-}$ [2]}. The assumption was made (and is justified by the present

results) that there is no negative cooperativity in the binding of the above inhibitors and the anionic ligands ADP-ribose and $\text{Pt}(\text{CN})_4^{2-}$. Proton uptake resulting from NADH binding was measured only with the enzyme · imidazole complex.

Equilibrium Measurements of Bipyridine Binding

Determination of the equilibrium dissociation constant for 2,2'-bipyridine binding to liver alcohol dehydrogenase was performed by spectrophotometric titration of the enzyme (about 10 μM) with bipyridine (0.1–5 mM). Absorbance changes at 312 nm were recorded with a Zeiss PM QII spectrophotometer, and the experimental data were evaluated as described by Sigman [13]. In some experiments, titrations were performed in the presence of at least 95% saturating concentrations of ADP-ribose (4 mM) or $\text{Pt}(\text{CN})_4^{2-}$ (10 mM).

Kinetic Measurements of Bipyridine Binding

The kinetics of 2,2'-bipyridine binding were examined by stop-flow techniques [14,15], using the rapid reaction equipment described previously [16]. The enzyme (about 10 μM) was reacted with varied concentrations of bipyridine, and apparent first-order rate constants for the exponential transients observed at 312 nm [14,15] were calculated by non-linear regression analysis [16].

RESULTS

Effect of Imidazole on the Binding of NADH, ADP-ribose, and $\text{Pt}(\text{CN})_4^{2-}$

It has been previously shown that binary-complex formation between liver alcohol dehydrogenase and anionic ligands such as NADH, AMP, or $\text{Pt}(\text{CN})_4^{2-}$ results in a pH-dependent uptake of protons from solution, half an active-site equivalent of protons being taken up at pH 9.2 [1]. This proton uptake derives from the pK_a 9.2 ionization step in Scheme 1 and is indicative of a corresponding pH dependence in the binding of the respective ligand to free enzyme. The data in Fig. 1 establish that an entirely analogous pH-dependent proton uptake occurs on the addition of (at least 95%) saturating concentrations of ADP-ribose to free enzyme, confirming that binary-complex formation with ADP-ribose exhibits a pK_a 9.2 dependence analogous to that of the binding of the above anionic ligands [2,17].

Similar titrimetric experiments were carried out over the pH range 8–10 in the presence of at least 95% saturating concentrations of imidazole. The results are given in Fig. 1. They establish that there is no significant uptake or release of protons on ternary-

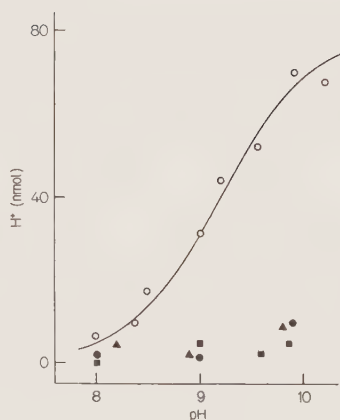
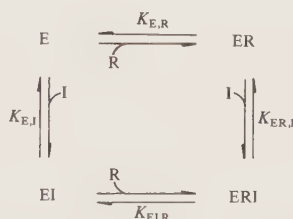


Fig. 1. Effect of pH on proton uptake resulting from complex formation at the coenzyme-binding site of liver alcohol dehydrogenase. Titrimetrically determined amount of protons withdrawn from the reaction medium (33 mM sodium sulphate solution) on reacting the enzyme (80 nmol) with 0.4 mM NADH ($\square$), 4 mM ADP-ribose ($\circ$), or 10 mM $\text{Pt}(\text{CN})_4^{2-}$ (Δ) at 25°C in the absence (open symbols) or presence (filled symbols) of 40 mM imidazole. The curve drawn corresponds to a monobasic dissociation function with a pK_a of 9.2



Scheme 2. Reaction scheme for ternary-complex formation between liver alcohol dehydrogenase (E), NADH (R), and imidazole (I)

complex formation between the enzyme · imidazole complex and NADH, ADP-ribose, or $\text{Pt}(\text{CN})_4^{2-}$. This observation provides direct evidence that complex formation with imidazole abolishes the pK_a 9.2 dependence of the binding of ligands which utilize the anion-binding part of the coenzyme-binding site.

Using the notation system of Theorell et al. [5], equilibrium dissociation constants for the binding steps leading to ternary-complex formation between enzyme (E), NADH (R), and imidazole (I) can be defined as in Scheme 2. These equilibrium constants are interrelated through

$$K_{\text{E,R}} \cdot K_{\text{ER,I}} = K_{\text{E,I}} \cdot K_{\text{EI,R}} \quad (1)$$

where $K_{\text{ER,I}}$ has been shown to be independent of pH over the range 6–10 [5,8]. The data in Fig. 1 indicate that there is no significant effect of pH on $K_{\text{EI,R}}$ between pH 8 and 10. According to Eqn (1),

this means that $K_{E,R}$ and $K_{E,I}$ must exhibit the same pH dependence over the latter pH range. The results in Fig. 1, therefore, provide the additional information that imidazole binding to free enzyme exhibits a pK_a 9.2 dependence analogous to that of the binding of NADH.

*Effect of Zinc-Chelating Reagents
on the Binding of ADP-ribose and $Pt(CN)_4^{2-}$*

The titrimetric experiments in the preceding section were repeated using binary enzyme · inhibitor complexes containing zinc-coordinating inhibitors [7] other than imidazole. No significant proton uptake was observed between pH 8 and 10 on the addition of at least 95% saturating concentrations of ADP-ribose or $Pt(CN)_4^{2-}$ to the binary complexes formed with 2,2'-bipyridine or 1,10-phenanthroline. These observations provide evidence that the pK_a 9.2 dependence of complex formation at the anion-binding part of the coenzyme-binding site is abolished also by the latter two-zinc-chelating reagents.

Effect of pH on the Binding of 2,2'-Bipyridine

The apparent dissociation equilibrium constant (K) for complex formation between liver alcohol dehydrogenase (E) and 2,2'-bipyridine (L) according to Eqn (2) was determined spectrophotometrically by titration



of the enzyme with ligand [13]. Experiments were performed at different pH between 7 and 10, absorbance changes due to complex formation being recorded at 312 nm. Regression analysis of the titration curves gave an essentially pH-independent value of $9800 \text{ M}^{-1} \text{ cm}^{-1}$ for the apparent difference absorption coefficient of the enzyme · bipyridine complex at this wavelength, whereas there was a pronounced effect of pH on the estimates of K obtained. The results in Fig. 2 show that the variation of K with pH can be well described in terms of the relationship

$$K = K^* \left(1 + \frac{K_a}{[H^+]} \right) \quad (3)$$

with $K^* = 320 (\pm 40) \mu\text{M}$ and $pK_a = 9.2 (\pm 0.2) \mu\text{M}$. Hence it may be concluded that bipyridine binding to free enzyme exhibits a pH dependence analogous to that of the binding of imidazole and anionic ligands such as NADH, ADP-ribose, and $Pt(CN)_4^{2-}$ [1,2,17].

Dissociation equilibrium constants were similarly determined between pH 7 and 10 for bipyridine binding to the enzyme · ADP-ribose and the enzyme · $Pt(CN)_4^{2-}$ complex. The results are included in Fig. 2 and confirm the previous report that there is no detectable cooperativity in the binding of bipyridine and ADP-ribose at pH 7 [13]. Above pH 9, however,



Fig. 2. Effect of pH on the dissociation equilibrium constant for complex formation between liver alcohol dehydrogenase and 2,2'-bipyridine. Equilibrium constants (K) determined spectrophotometrically at 25°C in the absence of anionic ligands (O) and in the presence of 4 mM ADP-ribose (●) or 10 mM $Pt(CN)_4^{2-}$ (▲). The curve drawn was calculated from Eqn (3) with $K^* = 320 \mu\text{M}$ and $pK_a = 9.2$.

complex formation with ADP-ribose significantly increase the affinity of the enzyme for bipyridine. The data in Fig. 2 establish that this positively cooperative effect of ADP-ribose binding derives from an elimination of the pK_a 9.2 dependence of bipyridine binding. It can, further, be seen from Fig. 2 that the pH dependence of bipyridine binding is similarly abolished by $Pt(CN)_4^{2-}$. This indicates that the effects observed are attributable to a cooperativity between bipyridine binding and complex formation at the anion-binding part of the coenzyme-binding site.

The data in Fig. 2, combined with the titrimetric results in the preceding sections, suggest that there is a general pH-dependent cooperative interrelationship between the binding of anionic ligands and the binding of reagents which combine to the catalytic zinc ion. Complex formation at the anion-binding subsite abolishes the pK_a 9.2 dependence of complex formation at the zinc ion, and *vice versa*.

Kinetics of 2,2'-Bipyridine Binding

The kinetics of bipyridine binding to liver alcohol dehydrogenase were examined by stop-flow techniques at different pH between 7 and 10. Apparent first-order rate constants (r) for the single-exponential transient observed at 312 nm on mixing enzyme with bipyridine were determined for a series of ligand concentrations at each pH. Fig. 3 shows some typical results which

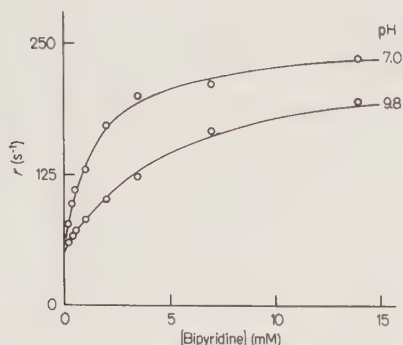


Fig. 3. Effect of ligand concentration and pH on the transient rate of complex formation between liver alcohol dehydrogenase and 2,2'-bipyridine. Apparent first-order rate constants (r) for the transient absorbance changes observed at 312 nm on reacting the enzyme (about 10 μM) at 25 °C with varied concentrations of bipyridine in 0.1 M ionic strength buffer solutions. Curves drawn were calculated from Eqn (4) with $r_{\infty} = 250 \text{ s}^{-1}$, $k_{\text{off}} = 50 \text{ s}^{-1}$, and k_{on} values according to Fig. 4

confirm previous reports [14,15] that the transient rate of complex formation approaches a saturation value (r_{∞}) with increasing concentrations of ligand (L). Quantitative evaluation of the experimental data was performed by non-linear regression analysis based on the results of Frolich et al. [15]. Estimates of r_{∞} and of the intercept (k_{off}) and initial slope (k_{on}) of the r vs [L] curve were calculated statistically by fitting the regression function

$$r = \frac{k_{\text{off}}(r_{\infty} - k_{\text{off}}) + r_{\infty}k_{\text{on}}[\text{L}]}{r_{\infty} - k_{\text{off}} + k_{\text{on}}[\text{L}]} \quad (4)$$

to the data obtained at each fixed pH. This gave $r_{\infty} = 250 (\pm 30) \text{ s}^{-1}$, $k_{\text{off}} = 50 (\pm 10) \text{ s}^{-1}$, and $k_{\text{on}} = 140 (\pm 20) \text{ s}^{-1} \text{ mM}^{-1}$ for the reaction at pH 7, which agrees well with the parameter values reported previously [15]. Calculated estimates of r_{∞} and k_{off} for the reaction at higher pH did not differ significantly from those obtained at pH 7 (cf. Fig. 3). The effect of pH on the parameter k_{on} is shown in Fig. 4 and can be satisfactorily described in terms of the relationship

$$k_{\text{on}} = \frac{k_{\text{on}}^*}{1 + \frac{K_a}{[\text{H}^+]}} \quad (5)$$

with $k_{\text{on}}^* = 140 \text{ s}^{-1} \text{ mM}^{-1}$ and $\text{p}K_a = 9.2$.

The $\text{p}K_a$ 9.2 dependence of k_{on} for bipyridine binding was found to be abolished by saturating concentrations of ADP-ribose and $\text{Pt}(\text{CN})_4^{2-}$, as might be expected from the results of the equilibrium binding studies described in the preceding section.

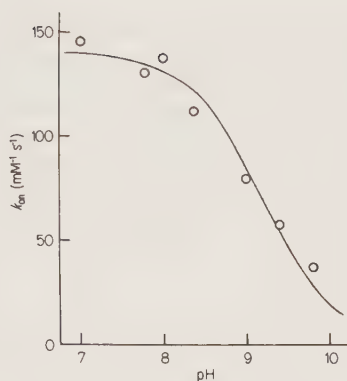


Fig. 4. Effect of pH on the parameter k_{on} for bipyridine binding to liver alcohol dehydrogenase. Conditions as in Fig. 3. The curve drawn was calculated from Eqn (5) with $k_{\text{on}}^* = 140 \text{ s}^{-1} \text{ mM}^{-1}$ and $\text{p}K_a = 9.2$

At low concentrations of bipyridine the saturation behaviour of r may be neglected and Eqn (4) reduces to

$$r = k_{\text{off}} + k_{\text{on}}[\text{L}]. \quad (6)$$

Under such conditions, the kinetic data in Fig. 3 and 4 can be interpreted in terms of Eqn (2) and indicate that the pH dependence of bipyridine binding derives from the on-velocity constant for complex formation. The saturation behaviour of r at high ligand concentrations, however, must be explained in view of an extended binding mechanism. Frolich et al. [15] have shown that the kinetics of bipyridine binding are consistent with the two-step mechanism



where EL^* represents a primary association complex and EL is the spectroscopically observable binary complex. Assuming that there is a rapid pre-equilibration between enzyme and ligand, parameters in Eqn (4) are related [15] to rate constants in Eqn (7) through

$$k_{\text{on}} = \frac{k_1 k_2}{k_{-1}} \quad (8)$$

$$k_{\text{off}} = k_{-2} \quad (9)$$

$$r_{\infty} = k_2 + k_{-2}. \quad (10)$$

When interpreted in terms of Eqns (7–10), the present kinetic data for bipyridine binding establish that there is no significant effect of pH on the rate constant k_2 and k_{-2} . The $\text{p}K_a$ 9.2 dependence of bipyridine binding must derive from the primary association step leading to formation of the intermediate EL^* in Eqn (7).

The same conclusion can be drawn from the equilibrium binding studies, which showed that the apparent absorption coefficient for the enzyme · bipyridine complex in Eqn (2) remains essentially unaffected by pH. The latter observation, when interpreted in view of Eqn (7), provides direct evidence that there is no significant effect of pH on the equilibrium between the chromophoric (EL) and the non-chromophoric (EL*) form of the enzyme · bipyridine complex.

DISCUSSION

Identity of the Ionizing Group Regulating Coenzyme Association

There are two identical subunits in the liver alcohol dehydrogenase molecule. Each subunit contains two zinc ions, one of which is catalytically essential. The catalytic zinc ion is bound by three protein ligands, a tetrahedral coordination to the metal being completed by a water molecule [6]. As was pointed out in the introduction, there seems to be little doubt that the pK_a 7.6 dependence of NAD^+ dissociation derives from ionization of this zinc-bound water molecule. It is recognized that the actual ionizing structure may involve zinc-bound water, hydrogen bonded to the hydroxyl group of Ser-48 and the imidazole group of His-51 [18]. For simplicity, however, the ionizing structure will be referred to as zinc-bound water in the following discussion.

Solution studies and crystallographic results have established that reagents such as imidazole, 1,10-phenanthroline, and 2,2'-bipyridine combine to the catalytic zinc ion [7]. The latter two reagents are bidentate ligands and form penta-coordinate complexes in which zinc-bound water has been displaced from the metal. The monodentate reagent imidazole might coordinate as a fifth ligand without displacement of zinc-bound water [19] although crystallographic data indicate that the water molecule is probably displaced by imidazole [7]. However that may be, the observation that imidazole abolishes the pK_a 7.6 dependence of NAD^+ dissociation [5] establishes that the ionization properties of zinc-bound water are drastically affected by imidazole binding. Our attention, therefore, has been drawn to the report that imidazole does not abolish the pH dependence of NADH binding [8]. A confirmation of the latter result would provide conclusive evidence that the pK_a 9.2 group cannot be identical with zinc-bound water. Conversely, a demonstration that the pK_a 9.2 dependence of coenzyme and anion binding is abolished by zinc-coordinating reagents would make it reasonable to identify the pK_a 9.2 group as zinc-bound water.

The present investigation provides a clear answer as concerns the effect of zinc-coordinating ligands on coenzyme and anion binding to the enzyme. The titrimetric data in Fig.1 establish that the pK_a 9.2 de-

pendence of NADH binding is completely eliminated on binary-complex formation with imidazole. Moreover, imidazole abolishes the pK_a 9.2 dependence of ADP-ribose and $Pt(CN)_4^{2-}$ binding (Fig.1), as would be expected from the previous conclusion that the pH dependence of NADH binding reflects an effect of pH on the anion-binding subsite [1]. The observation that the pK_a 9.2 dependence of complex formation at this subsite is abolished also by 1,10-phenanthroline and 2,2'-bipyridine, finally, demonstrates that the effects observed are related to a coordination of external ligands to the catalytic zinc ion and, most likely, to displacement of zinc-bound water (see below). These results strongly indicate that the pK_a 9.2 dependence of coenzyme association can be attributed to ionization of zinc-bound water.

An experimentally verifiable implication of the latter conclusion is that binding of the bidentate ligands 1,10-phenanthroline and 2,2'-bipyridine would be expected to exhibit a pK_a 9.2 dependence analogous to that of NADH binding. This follows from the fact that complex formation with the bidentate ligands involves displacement of zinc-bound water. The negatively charged hydroxyl ion can be anticipated to be considerably more firmly bound than neutral water to the positively charged zinc ion. Consequently, the bidentate ligands would be expected to exhibit a considerably higher affinity for the enzyme form in which zinc-bound water is in the neutral state. The data in Fig.2 establish that bipyridine binding to free enzyme shows such a dependence [Eqn (3)] on the ionization state of the pK_a 9.2 group. Reported equilibrium constants for 1,10-phenanthroline binding [12], similarly, exhibit a variation with pH which can be satisfactorily described by Eqn (3) using a pK_a of 9.2. These observations provide additional evidence that the pK_a 9.2 dependence of ligand binding to free enzyme derives from ionization of zinc-bound water.

Binding Mechanism for Complex Formation at the Catalytic Zinc Ion

The present conclusion that imidazole binding to free enzyme exhibits a pK_a 9.2 dependence analogous to that of the binding of NADH and zinc-chelating reagents seems to be consistent with and supported by previously reported data for binary-complex formation with imidazole at different pH [5,8,20]. For example, Reynolds et al. pointed out that the binding of imidazole and 1,10-phenanthroline is sevenfold weaker at pH 10 than at about pH 7 [20]. This is, indeed, what would be expected according to Eqn (3) if binding of the ligands is regulated by an ionizing group with a pK_a of 9.2. In our opinion, the simplest explanation for the observed pK_a 9.2 dependence of imidazole binding is that this monodentate ligand, by analogy to the bidentate ligands, combines to the

catalytic zinc ion with displacement of zinc-bound water. The latter conclusion has some bearing on the functional role of zinc-bound water in the catalytic reaction and calls for a more detailed discussion of the mechanism of complex formation at the catalytic zinc ion.

The data in Fig. 3 confirm previous reports that the association of bidentate ligands to the zinc ion shows a limiting rate of about 250 s^{-1} in the formation of the spectroscopically observable complex. Gilleland et al. [14] and Frolich et al. [15] interpreted this kinetic pattern in terms of a two-step binding mechanism [Eqn (7)]. The bidentate ligand was assumed to form an outer-sphere complex (EL*) in a rapid pre-equilibration process, subsequent formation of the chromophoric inner-sphere complex (EL) being rate-limited by dissociation of the zinc-bound water molecule. Frolich et al. showed that there is no similar detectable limitation of the rate of association of monodentate ligands, which led them to suggest that such ligands (and substrates, by analogy) combine to zinc without any displacement of zinc-bound water. Hence they concluded that a penta-coordinate water molecule could play an essential role in the proton transfer reaction accompanying catalysis, an idea which has been recently advanced by Dworschack et al. [21].

We agree that it is reasonable to interpret the kinetic data in Fig. 3 in terms of a two-step binding mechanism. The observed limiting rate of about 250 s^{-1} , however, seems to be far too low to represent the dissociation rate of a zinc-bound water molecule [22, 23]. The present observation that the pK_a 9.2 dependence of bipyridine binding derives from the primary association step in Eqn (7), while there is no significant effect of pH on the subsequent rate-limiting step, suggests that there is an alternative and more plausible explanation for the limitation of the association rate of zinc-chelating reagents. As was pointed out in the preceding section, the effect of pH on the binding of such reagents is likely to reflect the firmer binding of the zinc-bound hydroxyl ion compared to the neutral water molecule. Desorption of the water molecule, therefore, would be expected to take place in the reaction step which exhibits the experimentally observed dependence on the protonation state of zinc-bound water. This means that displacement of zinc-bound water most likely occurs in the primary association step in Eqn (7), probably through a monodentate inner-sphere coordination of the ligand to zinc. The subsequent rate-limiting step can then be envisaged to represent bidentate chelate formation through penta-coordination of the ligand. The latter process would be expected to be comparatively slow, since it changes the coordination number of the metal and hence is likely to involve readjustments of the entire coordination geometry.

The implication of such an interpretation of the present and previously reported data for complex formation at the catalytic zinc ion is that bidentate ligands exhibit a limiting rate in their association, not because they displace zinc-bound water, but because they form penta-coordinate complexes with a metal which has a coordination number of four prior to binding of the ligand. The fact that neither substrates [24], nor monodentate substrate analogues [15], show such a limiting rate in their association, therefore, would seem to indicate that they bind without changing the coordination number of zinc, i.e. with displacement of zinc-bound water. The present observation that binding of the monodentate ligand imidazole exhibits a pK_a 9.2 dependence lends strong support to the latter idea.

Interpreted along the above lines, the data of Frolich et al. [15] actually provide evidence against the idea that penta-coordinate complexes are formed on the binding of monodentate ligands to the catalytic zinc ion. It has been concluded from recent kinetic data that the effect of pH on the binding of substrates and monodentate substrate analogues can be best understood in terms of complex formation with displacement of zinc-bound water [25, 26]. The present results reinforce that conclusion.

Interrelationship between Ionization of Zinc-Bound Water and the Binding of Anionic Ligands

Crystallographic studies of liver alcohol dehydrogenase [19, 27, 28] have shown that the enzyme subunit is divided into two domains which are separated by a cleft containing a deep pocket where the catalytic reaction occurs. One of the domains binds the coenzyme and accommodates its nicotinamide portion in the active-site pocket. The catalytic zinc ion is bound at the bottom of the pocket by protein ligands from the second domain (Cys-46, Cys-174 and His-67). The anion $\text{Pt}(\text{CN})_4^{2-}$ is bound at the same position (about 1 nm from the catalytic zinc ion) as the pyrophosphate group of coenzyme and ADP-ribose. The side-chain of Arg-47 is rigidly fixed in the enzyme $\cdot \text{Pt}(\text{CN})_4^{2-}$ complex and bridges the cleft between the two domains of the subunit to interact with the anionic ligand. This indicates that the positively charged guanidinium group of Arg-47 forms an essential part of a general anion-binding site which functions as a binding site for the pyrophosphate group of the coenzyme [28]. Solution studies of various kinds have provided strong evidence in favour of this idea [20, 29, 31].

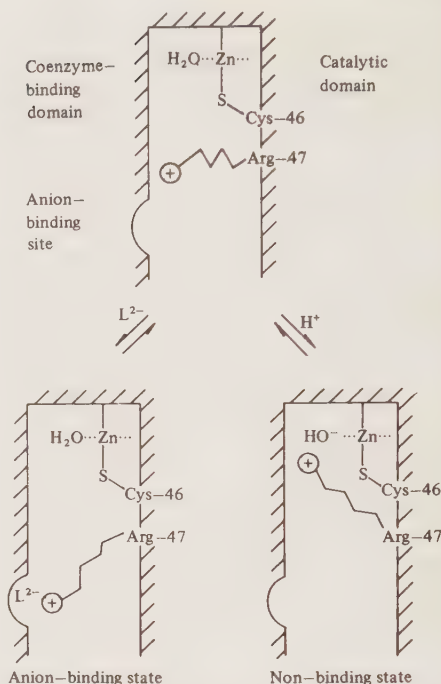
As was mentioned in the introduction, the pK_a 9.2 dependence of coenzyme binding is likely to derive from effects of pH on complex formation at this anion-binding site. In contrast to zinc-coordinating reagents, however, anionic ligands such as NADH,

ADP-ribose, and $\text{Pt}(\text{CN})_4^{2-}$ form binary complexes in which the water molecule at the catalytic zinc ion remains bound to the metal [6, 27, 28]. Our conclusion that the pK_a 9.2 dependence of coenzyme binding is attributable to deprotonation of zinc-bound water, therefore, calls for a discussion of the mechanism by which the ionization state of this group affects the binding of anionic ligands.

From a kinetic point of view, the effect of pH on complex formation at the anion-binding site can be characterized as a positively cooperative binding of protons and anionic ligands to the enzyme. The fact that ligands of opposite charge are involved suggests that this cooperativity is basically of electrostatic origin; it seems to be the appearance of a negatively charged ligand at the catalytic zinc atom which prevents the binding of anionic ligands at high pH. Qualitatively, it is obvious that ionization of zinc-bound water must give rise to electrostatic interactions resulting in a thermodynamic destabilization of the complex formed with an anionic ligand. Considering the distance between the (ultimately) interacting charges, there is no doubt that such electrostatic interactions could be strong enough to account also for the observed quantitative effects of pH on the binding of anionic ligands.

From a kinetic point of view, however, it seems inadequate to attribute the pK_a 9.2 dependence of coenzyme binding to a direct repulsive interaction between the zinc-bound hydroxyl ion and the pyrophosphate group of enzyme-bound coenzyme. This follows from the fact that the pK_a 9.2 dependence derives from the rate of coenzyme association [1] and hence (according to the simplest interpretation) reflects the binding properties of free enzyme. It has been previously inferred from the latter observation that ionization of the pK_a 9.2 group may trigger a conformational change which renders the enzyme unable to interact properly with anionic ligands [1]. The present identification of the pK_a 9.2 group as zinc-bound water makes it possible to state a specific (minor) change in enzyme conformation which would have such an effect and which is likely to be triggered by the formation of a zinc-bound hydroxyl ion (Scheme 3).

As has been pointed out previously [28], there is crystallographic and kinetic evidence indicating that the side-chain of Arg-47 is quite mobile. It occupies no unique position in free enzyme, but bridges the cleft between the two domains of the enzyme subunit to interact with negatively charged ligands at the coenzyme-binding site (the binding state in Scheme 3). It can also combine to an anion such as iodoacetate and transfer this alkylating reagent into proper position for an attack on the sulphur of Cys-46, which is coordinated to the catalytic zinc ion. Examination of the structure of the enzyme [27] confirms that it is possible to have the guanidinium group of Arg-47



Scheme 3. Competitive salt bridge formations proposed to account for the cooperative interrelationship between ionization of zinc-bound water and complex formation with anionic ligands at the coenzyme-binding site of liver alcohol dehydrogenase

positioned in proximity to the catalytic zinc ion through conformational changes of the side-chain carrying this group. Ionization of zinc-bound water, therefore, is likely to trigger such conformational changes through electrostatic interactions, i.e. to result in formation of a salt bridge between the zinc-bound hydroxyl ion and the guanidinium group of Arg-47 (the non-binding state in Scheme 3). According to this idea, there is a competition between the zinc-bound hydroxyl group and external anionic ligands for the guanidinium group of Arg-47. This explains why ionization of zinc-bound water leads to a thermodynamic destabilization of the complexes formed with anionic ligands. It also explains why this effect can be attributed to the binding properties of free enzyme. The engagement of the main constituent of the general anion-binding site in an internal salt bridge, obviously, must render the enzyme unable to interact properly with external anionic ligands.

If protonation of the zinc-bound hydroxyl ion facilitates complex formation at the anion-binding site, the binding of anionic ligands must facilitate protonation of the zinc-bound hydroxyl ion to the same

extent. In other words, the pK_a of zinc-bound water would be expected to increase on complex formation at the anion-binding site. This is consistent with and explains the data in Fig. 2, which show that the pK_a 9.2 dependence of bipyridine binding is abolished on binary-complex formation with ADP-ribose and $Pt(CN)_4^{2-}$. The observation that there is no effect of pH on imidazole binding to the enzyme $\cdot$ NADH complex [5,8] indicates that the pK_a 9.2 dependence of ligand binding to zinc is similarly abolished by NADH. Furthermore, titrimetric experiments have provided direct evidence that the pK_a of the ionizing group controlling coenzyme association and anion binding is perturbed from 9.2 to a value above 10 in the binary complexes formed with NADH, AMP and $Pt(CN)_4^{2-}$ [1].

This anion-induced perturbation of the pK_a of zinc-bound water can be well understood in view of Scheme 3. In the non-binding conformational state of the enzyme, ionization of zinc-bound water is facilitated by the stabilizing interaction with the guanidinium group of Arg-47. This explains why the water molecule exhibits a pK_a as low as 9.2 despite the fact that it is coordinated to a zinc ion which is bound by two negatively charged protein ligands (Cys-46 and Cys-174). Complex formation with ligands such as NADH, ADP-ribose, AMP, and $Pt(CN)_4^{2-}$ converts the enzyme into the binding conformational state in Scheme 3. In the latter state, the side-chain of Arg-47 is engaged in salt bridge formation at the anion-binding site and cannot participate in stabilization of the zinc-bound hydroxyl ion. This explains why the pK_a of zinc-bound water is higher in the complexes formed with anionic ligands than in free enzyme. The pH-dependent cooperative effects of anion-binding and complex formation at the catalytic zinc ion can be rationalized in a similar way.

Admittedly, the structural interrelationship between ionization of zinc-bound water and complex formation at the anion-binding site may be more complex than indicated in Scheme 3. Electrostatic interactions are of considerable strength, and the appearance of a charged group on the enzyme surface can be anticipated to result in minor conformational changes of a large number of surrounding polar or ionized groups. Interactions with the protein environment (including the side-chain of Arg-47) might be different with different anionic ligands, and the possibility that the negative charge on the zinc-bound hydroxyl ion might be partly delocalized over an extended hydrogen-bonded structure [18] adds to the complexity of the system considered. Nevertheless, competitive breaking of a salt bridge between the guanidinium group of Arg-47 and the zinc-bound hydroxyl ion would be expected to provide a major energetic contribution to the observed effect of anionic ligands on the pK_a of zinc-bound water, which justifies

description of the system in terms of Scheme 3. It should be mentioned in this context that the side-chain of Arg-47 must be assumed to interact also with anions of the buffer solution. This means that the composition of the reaction medium may affect the pK_a of zinc-bound water, which could explain minor differences between reported pK_a values for kinetically significant ionization steps now suggested to involve zinc-bound water.

Summing up the above discussion, it seems reasonable to assume that the pK_a 9.2 dependence of coenzyme association to liver alcohol dehydrogenase derives from ionization of zinc-bound water, the zinc-bound hydroxyl ion being stabilized in free enzyme through salt bridge formation with the side-chain of Arg-47. Breaking of this salt bridge on coenzyme binding increases the pK_a of zinc-bound water to a value above 10 in the enzyme $\cdot$ NADH complex, which accounts for the lack of effect of pH on the NADH dissociation rate between pH 6 and 10. This pK_a perturbation can be ultimately attributed to destabilization of the zinc-bound hydroxyl ion through electrostatic interactions with the negatively charged pyrophosphate group of the coenzyme. In the enzyme $\cdot$ NAD⁺ complex, the corresponding repulsive destabilization of the zinc-bound hydroxyl ion is counteracted by electrostatic interaction with the positively charged nicotinamide ring of the coenzyme. Since the positive charge on enzyme-bound NAD⁺ is located in much closer proximity to the catalytic zinc atom than is the negatively charged pyrophosphate group of the coenzyme, NAD⁺ binding results in a net thermodynamic stabilization of the zinc-bound hydroxyl ion. This accounts for the decrease in pK_a of zinc-bound water to a value of 7.6 in the enzyme $\cdot$ NAD⁺ complex, and hence for the pK_a 7.6 dependence of the rate of coenzyme dissociation from the latter complex.

The present investigation, therefore, leads us to conclude that all main effects of pH on coenzyme binding between pH 6 and 10 can be explained in terms of the ionization state of zinc-bound water (this conclusion is consistent with and supported by the report of Coleman et al. [32] that there is no effect of pH on NADH or NAD⁺ binding to zinc-free enzyme). Furthermore, the pH dependence of anion-binding in general can be well understood in such terms, as can the effect of pH on substrate [25,26] and inhibitor binding to the catalytic zinc ion and the pH-dependent cooperative interrelationship between anion-binding and complex formation at the zinc ion. It would, in fact, seem extremely difficult to provide any reasonable explanation for the binding data now reported without assuming that the pK_a 9.2 dependence of ligand binding to liver alcohol dehydrogenase reflects the ionization state of zinc-bound water.

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Effect of NADH on the pK_a of Zinc-Bound Water in Liver Alcohol Dehydrogenase

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Equilibrium constants for coenzyme binding to liver alcohol dehydrogenase have been determined over the pH range 10–12 by pH-jump stop-flow techniques. The binding of NADH or NAD^+ requires the protonated form of an ionizing group (distinct from zinc-bound water) with a pK_a of 10.4. Complex formation with NADH exhibits an additional dependence on the protonation state of an ionizing group with a pK_a of 11.2. The binding of *trans*-*N,N*-dimethylaminocinnamaldehyde to the enzyme · NADH complex is prevented by ionization of the latter group.

It is concluded from these results that the pK_a -11.2-dependence of NADH binding most likely derives from ionization of the water molecule bound at the catalytic zinc ion of the enzyme subunit. The pK_a value of 11.2 thus assigned to zinc-bound water in the enzyme · NADH complex appears to be typical for an aquo ligand in the inner-sphere ligand field provided by the zinc-binding amino acid residues in liver alcohol dehydrogenase. This means that the pK_a of metal-bound water in zinc-containing enzymes can be assumed to correlate primarily with the number of negatively charged protein ligands coordinated by the active-site zinc ion.

Substrate and coenzyme binding to liver alcohol dehydrogenase appears to be drastically affected by ionization of the water molecule which is bound at the catalytic zinc ion of the enzyme subunit [1–6]. In a recent investigation of the pH dependence of ligand binding at the catalytic zinc ion [7] we concluded that zinc-bound water exhibits a pK_a of 9.2 in free enzyme, decreasing to 7.6 in the enzyme · NAD^+ complex and increasing to a value above 10 in the enzyme · NADH complex. We have now attempted to obtain more detailed information about the effect of complex formation with NADH on the pK_a of zinc-bound water by examination of the effect of pH on NADH binding at pH values above 10. The results described in the present paper show that complex formation between enzyme and NADH is regulated by a proton dissociation equilibrium with a pK_a of 11.2, while there is no corresponding effect of pH on NAD^+ binding over the examined pH range (10–12). We propose that the observed pK_a -11.2-dependence of NADH binding derives from ionization of zinc-bound water in the binary complex formed. Equilibrium data for aldehyde binding to the enzyme · NADH complex are reported which lend strong support to the latter proposal.

Enzyme. Liver alcohol dehydrogenase or alcohol:NAD⁺ oxidoreductase (EC 1.1.1.1).

EXPERIMENTAL PROCEDURE

Materials

Crystalline horse liver alcohol dehydrogenase from Boehringer (Mannheim) was further purified as described by Bernhard et al. [8]. Unbuffered enzyme solutions were prepared using 33 mM sodium sulphate for elution of the enzyme in the column chromatographic purification step. Enzyme concentrations were determined fluorimetrically by titration with NADH in the presence of isobutyramide [9] and are reported throughout as active-site concentrations.

The coenzymes NADH and NAD^+ (Sigma Chemical Corp.; grade III quality) were purified as described by Gurr et al. [10]. *trans*-*N,N*-Dimethylaminocinnamaldehyde was obtained from BDH Chemicals (Poole) and used without further purification.

Spectrophotometric Measurements and Data Processing

All experiments in the present investigation were performed at 25°C in 0.1 M ionic strength phosphate buffer solutions adjusted to the appropriate pH between 10 and 12. Spectrophotometric measurements were made by stop-flow techniques, using the rapid-reaction equipment described previously [11]. Rates

and amplitudes of exponential transients observed were estimated by non-linear regression analysis as detailed previously [11]. Similar regression methods were used to obtain statistical estimates of dissociation constants in the equilibrium binding studies reported.

Enzyme Inactivation Rates

Liver alcohol dehydrogenase (10 μM) was incubated in 0.1 M ionic strength phosphate buffer at different fixed pH between 10 and 12.5. Samples were withdrawn after varied periods of time and tested for enzyme activity by steady-state rate determinations, using NADH (0.5 mM) and acetaldehyde (5 mM) as the substrates. Inactivation of the enzyme was found to proceed by apparent first-order kinetics at each pH tested, and the corresponding inactivation rate constants were determined by non-linear regression analysis.

NADH Binding

The binding of NADH to liver alcohol dehydrogenase was examined by pH-jump relaxation methods. The enzyme (about 20 μM) was preincubated in 33 mM sodium sulphate solution at pH 7 with varied concentrations of NADH (40–400 μM), high enough to be considered as fully saturating at this pH [1–3]. The unbuffered solution containing the enzyme · NADH complex was then mixed in the stop-flow apparatus with an equal volume of alkaline phosphate buffer to give a reaction solution of 0.1 M ionic strength and appropriate pH between 10 and 12. Due to the weaker binding of NADH at high pH [1–3], the rapid increase in pH initiated a more-or-less extensive dissociation of the binary complex to attain the new equilibrium concentration. The rate and amplitude of the exponential transient governing this relaxation process was determined from the absorbance changes observed at 355 nm. The pH of the reaction solution after mixing could be predicted from pH measurements made in pilot experiments and was routinely checked immediately after the reaction had been completed.

NAD⁺ Binding

The binding of NAD⁺ to liver alcohol dehydrogenase was examined by pH-jump methods under standard pre-mixing conditions. An about 10 μM solution of enzyme in 33 mM sodium sulphate (pH 7) was mixed in the stop-flow apparatus with alkaline buffer containing varied concentrations of NAD⁺ (40–1000 μM) to give a reaction solution of 0.1 M ionic strength and appropriate pH between 10 and 12. The rate and amplitude of the exponential transient governing formation of the enzyme · NAD⁺ complex was determined from the absorbance changes observed at 281 nm.

Complementary determination of the NAD⁺ dissociation rate constant at pH 10–11 were made by displacement methods as described previously [3].

Aldehyde Binding

Liver alcohol dehydrogenase (2–5 μM) was preincubated with varied concentrations of *trans*-*N,N*-dimethylaminocinnamaldehyde (5–300 μM) in 33 mM sodium sulphate solution at pH 7. The unbuffered solution containing enzyme and aldehyde was then mixed in the stop-flow apparatus with an equal volume of alkaline buffer containing varied concentrations of NADH (0.2–10 mM) to give a reaction solution of 0.1 M ionic strength and appropriate pH between 10 and 12. The amplitude of the exponential transient reflecting formation of the chromophoric enzyme · NADH · aldehyde complex was determined from the absorbance changes observed at 464 nm [12].

RESULTS

Theoretical Background

Ligand (L) binding to an enzyme (E) containing identical and independent binding sites can be described in terms of the reaction scheme



If the ligand is present in large excess to the total concentration (c_E) of enzymic sites, it follows from elementary kinetic theory [13, 14] that transient changes in the concentration of binary complex in Eqn (1) will conform to the relationship

$$[EL] = \frac{k_{\text{on}}[L]c_E}{r} - A \cdot e^{-rt}, \quad (2)$$

where the amplitude (A) and rate parameter (r) for the exponential transient are given by

$$r = k_{\text{off}} + k_{\text{on}}[L] \quad (3)$$

$$A = \frac{k_{\text{on}}[L]c_E}{r} - [EL]_0. \quad (4)$$

$[EL]_0$ in Eqn (4) stands for the concentration of binary complex at zero reaction time ($t = 0$). When $[EL]_0 = 0$, Eqn (4) may be written as

$$A = \frac{c_E}{1 + \frac{K_{E,L}}{[L]}}, \quad (5)$$

where $K_{E,L}$ denotes the equilibrium dissociation constant defined by

$$K_{E,L} = \frac{k_{\text{off}}}{k_{\text{on}}}. \quad (6)$$

When $[EL]_0 = c_E$ we have

$$A = \frac{c_E}{1 + \frac{[L]}{K_{E,L}}} \quad (7)$$

Eqns (5–7) show that direct estimates of $K_{E,L}$ can be obtained from measurements of A as a function of $[L]$. This corresponds to standard equilibrium methods for dissociation constant determinations by spectrophotometric titration of the enzyme with ligand. Indirect kinetic estimates of $K_{E,L}$ can according to Eqns (3) and (6), be obtained by determination of k_{on} and k_{off} from measurements of r as a function of $[L]$. Both methods for determination of $K_{E,L}$ were applied in the present investigation. Kinetic and equilibrium estimates of $K_{E,L}$ thus obtained were found to agree within experimental precision.

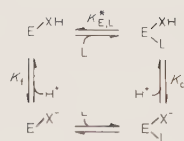
The effect of an ionizing enzymic group on ligand binding according to Eqn (1) can be interpreted in terms of the extended reaction system in Scheme 1. Equilibrium treatment of this system shows that the effect of pH on $K_{E,L}$ deriving from ionization of the enzymic group can be expressed in terms of two ionization functions as

$$K_{E,L} = K_{E,L}^* \cdot \frac{1 + \frac{K_f}{[H^+]}}{1 + \frac{K_c}{[H^+]}} \quad (8)$$

where K_f and K_c denote acid dissociation constants for the ionizing group in, respectively, free enzyme and the binary enzyme · ligand complex.

Stability of Reactants

The stability of liver alcohol dehydrogenase, NADH, NAD^+ , and dimethylaminocinnamaldehyde in phosphate buffer at pH values between 10 and 12 was checked by kinetic and spectroscopic control experiments. No significant decomposition of the low-molecular-weight reactants occurred over the period of time (15 min) considered in these experiments. The enzyme, however, was found to loose activity at a significant rate in a pH-dependent apparently first-order inactivation process. Table 1 shows that the inactivation rate constant varies in approximate proportion to the three-halves power of the hydroxyl ion concentration, exhibiting a value corresponding to a half-time of 1 min at pH 12. To avoid extensive inactivation of the enzyme, therefore, kinetic and equilibrium measurements now reported were performed by pH-jump methods using stop-flow techniques. Absorbance data required for determination of binding constants could be obtained within less than 10 s by such methods, which made it possible to carry out binding studies up to pH 12 without any detectable interference from enzyme inactivation.



Scheme 1. Reaction scheme considered for description of the effect on ligand binding of an ionizing enzymic group. $K_{E,L}^*$ denotes the equilibrium constant for ligand (L) dissociation from the binary complex formed with the protonated form (E^{XH}) of the enzyme. K_f and K_c stand for the acid dissociation constant of the ionizing group in, respectively, free enzyme and the binary enzyme · ligand complex

Table 1. Rate of inactivation of liver alcohol dehydrogenase in alkaline solution

Apparent first-order rate constants (k) for the pH-dependent loss of enzyme activity in 0.1 M ionic strength phosphate buffer at 25 °C

pH	k min ⁻¹	$k \cdot [OH^-]^{-1.5}$ min ⁻¹ M ^{1.5}
10.0	0.0008	800
10.5	0.0029	500
11.0	0.016	900
11.5	0.10	600
12.0	0.71	700
12.5	4.6	800

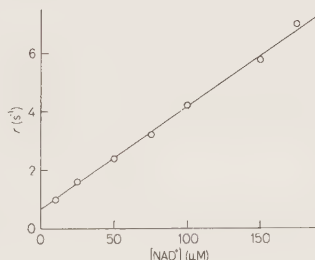


Fig. 1. Dependence on coenzyme concentration of the rate of the transient governing complex formation between NAD^+ and liver alcohol dehydrogenase. Apparent first-order rate constants (r) calculated from the transient observed at 281 nm on reacting the enzyme (5 μ M) with varied concentrations of NAD^+ in 0.1 M ionic strength phosphate buffer (pH 11.0) at 25 °C

NAD^+ Binding

The transient-state kinetics of NAD^+ binding to liver alcohol dehydrogenase were examined over the pH range 10–12 under such conditions ($[EL]_0 = 0$) that Eqns (2–5) apply. The typical results in Fig. 1 show the linear dependence on NAD^+ concentration of the rate parameter (r) for the exponential transient governing complex formation at pH 11.0. A fit of Eqn (3) to the data in Fig. 1 gave $k_{off} = 0.70 (\pm 0.06) s^{-1}$ and $k_{on} = 35 (\pm 3) s^{-1} mM^{-1}$, which corresponds to a

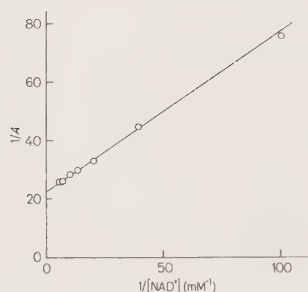


Fig. 2. Dependence on coenzyme concentration of the amplitude of the transient governing complex formation between NAD^+ and liver alcohol dehydrogenase. Conditions as in Fig. 1. Amplitudes (A) are given as absorbances

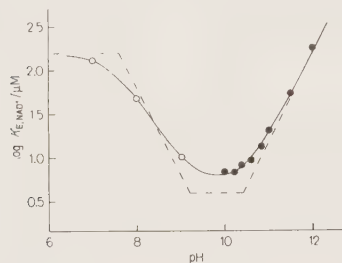


Fig. 3. Effect of pH on the equilibrium constant for NAD^+ dissociation from the binary complex formed with liver alcohol dehydrogenase. Dissociation constants ($K_{\text{E,NAD}^+}$) now determined (●) at 25 °C in 0.1 M ionic strength phosphate buffer, pH 10–12. Previously reported data (○) for the pH range 7–10 [3] are included for comparison. The curve drawn was calculated from Eqn (9) using $\text{p}K_{\text{c}} = 7.6$, $\text{p}K_{\text{f}} = 9.2$, $\text{p}K_{\text{f}0} = 10.4$, and $K_{\text{E,NAD}^+}^* = 160 \mu\text{M}$

value of $20 \mu\text{M}$ for the equilibrium dissociation constant $K_{\text{E,NAD}^+}$. Fig. 2 shows the coenzyme concentration dependence of the amplitude of the same transient. Application of Eqn (5) to the data in Fig. 2 gave $K_{\text{E,NAD}^+} = 24 (\pm 4) \mu\text{M}$. The arithmetic mean ($22 \mu\text{M}$) of the two values calculated for the dissociation constant was taken to represent the magnitude of $K_{\text{E,NAD}^+}$ at pH 11.0. The estimates of $K_{\text{E,NAD}^+}$ thus obtained at different pH between 11 and 12 are given in Fig. 3.

The on-velocity constant for NAD^+ binding to the enzyme was similarly determined between pH 10 and 11. Data such as those in Fig. 1 usually failed to provide satisfactorily precise estimates of k_{off} over the latter pH range. Values of $K_{\text{E,NAD}^+}$ below pH 11 (Fig. 3), therefore, were calculated from Eqn (6) using estimates of k_{off} obtained by conventional displacement methods [3].

Previously reported data for the binding of NAD^+ below pH 10 have been found to conform to Eqn (8)

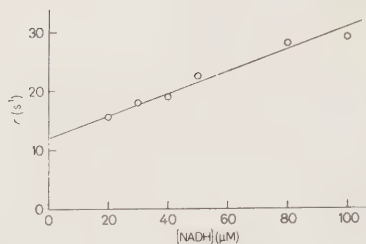


Fig. 4. Dependence on coenzyme concentration of the rate of the transient governing complex formation between NADH and liver alcohol dehydrogenase. Apparent first-order rate constants (r) calculated from the transient observed at 335 nm on relaxation of the enzyme $\cdot \text{NADH}$ complex ($10 \mu\text{M}$) at 25 °C in 0.1 M ionic strength phosphate buffer (pH 10.8) containing varied concentrations of NADH

with $K_{\text{E,NAD}^+}^* = 160 \mu\text{M}$, $\text{p}K_{\text{c}} = 7.6$, and $\text{p}K_{\text{f}} = 9.2$ [3]. The results in Fig. 3 indicate that NAD^+ binding is affected by an additional ionization step with a $\text{p}K_{\text{a}}$ above 10. The increasing magnitude of $K_{\text{E,NAD}^+}$ at high pH can be closely described by the inclusion of a third ionization function in Eqn (8) to give

$$K_{\text{E,NAD}^+} = K_{\text{E,NAD}^+}^* \cdot \frac{\left(1 + \frac{K_{\text{f}}}{[\text{H}^+]}\right) \left(1 + \frac{K_{\text{f}0}}{[\text{H}^+]}\right)}{1 + \frac{K_{\text{c}}}{[\text{H}^+]}} \quad (9)$$

Using the above estimates of $K_{\text{E,NAD}^+}^*$, $\text{p}K_{\text{c}}$, and $\text{p}K_{\text{f}}$, a fit of Eqn (9) to the present observations of $K_{\text{E,NAD}^+}$ gave $\text{p}K_{\text{f}0} = 10.4 (\pm 0.2)$.

NADH Binding

The transient-state kinetics of NADH binding to liver alcohol dehydrogenase over the pH range 10–12 were similarly examined, except that pre-mixing conditions were such ($[\text{EL}]_0 = c_{\text{E}}$) that Eqn (7) applies. Use was made of the fact that NADH is bound firmly to the enzyme at neutral pH but considerably more weakly at alkaline pH [1–3]. Under such conditions, the rapid increase in pH of a neutral solution containing the enzyme $\cdot \text{NADH}$ complex will initiate a dissociation of the complex which can be monitored spectrophotometrically at 335 nm.

As predicted by theory, the exponential transient governing this relaxation process was found to conform to Eqns (2–4). The typical results in Fig. 4, for example, show the linear dependence on NADH concentration of the rate parameter (r) for the transient observed at pH 10.8. The equilibrium dissociation constant ($K_{\text{E,NADH}}$) for binary-complex formation with reduced coenzyme was determined by methods anal-

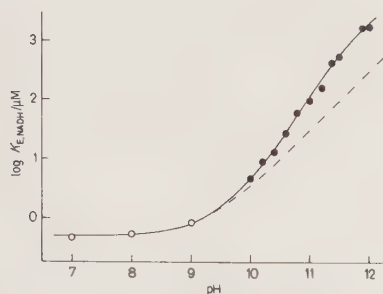


Fig. 5. Effect of pH on the equilibrium constant for NADH dissociation from the binary complex formed with liver alcohol dehydrogenase. Dissociation constants ($K_{E,NADH}$) now determined (●) at 25°C in 0.1 M ionic strength phosphate buffer, pH 10–12. Previously reported data (○) for the pH range 7–10 [3] are included for comparison. The dashed curve corresponds to a single ionization function with a pK_a of 9.2. The full curve was calculated from Eqn (10) using $pK_f = 9.2$, $pK_{10} = 10.4$, $pK_{11} = 11.2$, and $K_{E,NADH}^* = 0.5 \mu\text{M}$

ogous to those described above for NAD^+ binding. The estimates of $K_{E,NADH}$ obtained at different pH between 10 and 12 are given in Fig. 5.

It has been shown previously that complex formation between enzyme and NADH exhibits a pK_a -9.2-dependence analogous to that of NAD^+ binding [3]. The experimentally observed magnitude of $K_{E,NADH}$ at pH values above 10 (Fig. 5) is much too large, however, to reflect the effect of a single ionization step with a pK_a of 9.2. This might indicate that ionization of the pK_a -10.4-group controlling NAD^+ binding (Fig. 3) has a similar destabilizing effect on the binding of NADH. Regression analysis of the data in Fig. 5 provided evidence that such is the case, but also indicated that a third ionization function with a pK_a (pK_{11}) close to 11 limits the magnitude of $K_{E,NADH}$ according to the relationship

$$K_{E,NADH} = K_{E,NADH}^* \cdot \frac{\left(1 + \frac{K_f}{[H^+]}\right) \left(1 + \frac{K_{10}}{[H^+]}\right)}{1 + \frac{K_{11}}{[H^+]}} \quad (10)$$

This is illustrated in Fig. 6, which shows the factor by which experimental observations of $K_{E,NADH}$ deviate from the values calculated from Eqn (10) with the assumption that the dissociation constant is affected only by the two numerator functions with $pK_f = 9.2$ and $pK_{10} = 10.4$. The significance of the pK_{10} function is confirmed by the absence of a decrease in magnitude of the deviation factor attributable to a pK_a of 10.4. The significance of the denominator function in Eqn (10) is established by the steady increase in magnitude of the deviation factor, which corresponds to an ionization

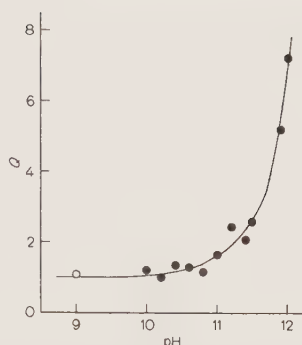


Fig. 6. Residual analysis of the pH dependence of NADH binding. Deviation factors (Q) for the residuals obtained on fitting Eqn (10) to the data in Fig. 5 with the assumption that $pK_f = 9.2$, $pK_{10} = 10.4$, and $K_{11} = 0$. The curve drawn corresponds to an ionization function with a pK_a of 11.2

function with a pK_a of $11.2 (\pm 0.2)$. The curve drawn in Fig. 5 indicates that Eqn (10) can be excellently fitted to the observed values of $K_{E,NADH}$ using $pK_f = 9.2$, $pK_{10} = 10.4$, $pK_{11} = 11.2$, and the previously reported [3] estimate $K_{E,NADH}^* = 0.5 \mu\text{M}$.

It may be concluded from these results that the binding of both oxidized and reduced coenzyme to liver alcohol dehydrogenase is destabilized by an ionization step with a pK_a of 10.4. Complex formation with NADH, but not with NAD^+ , exhibits an additional dependence on a stabilizing ionization step with a pK_a of 11.2.

Aldehyde Binding

trans-N,N-Dimethylaminocinnamaldehyde is known to form a tight ternary complex with liver alcohol dehydrogenase and NADH [12, 15]. This complex is stable at high pH and exhibits a characteristic absorption band centered at 464 nm. The equilibrium constant ($K_{E \cdot NADH, ald}$) for aldehyde dissociation from the ternary complex, therefore, can be readily determined from the absorbance changes reflecting ternary-complex formation in solutions containing varied concentrations of aldehyde and saturating concentrations of NADH [12].

Such experiments were performed by pH-jump methods over the pH range 10–12. Since the combination of dimethylaminocinnamaldehyde to the enzyme · NADH complex is too rapid to be conveniently monitored by stop-flow techniques [12], amplitudes reflecting ternary-complex formation were measured from the transient observed at 464 nm on the addition of NADH to a solution of enzyme pre-mixed with the aldehyde. The typical results in Fig. 7 illustrate the hyperbolic dependence on NADH concentration of

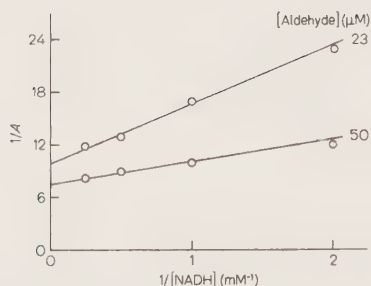


Fig. 7. Dependence on NADH concentration of the amplitude of the transient governing ternary-complex formation between liver alcohol dehydrogenase, NADH, and trans-N,N-dimethylaminocinnamaldehyde. Amplitudes (A), as absorbances, calculated for the transient observed at 464 nm on reacting enzyme (2 μ M), preincubated with fixed concentrations of the aldehyde, with varied concentrations of NADH in 0.1 M ionic strength phosphate buffer (pH 11.5) at 25 $^{\circ}$ C

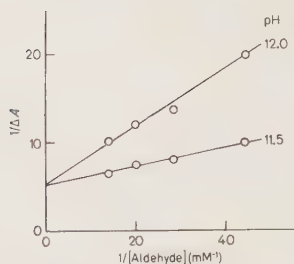


Fig. 8. Dependence on aldehyde concentration of the absorbance change reflecting trans-N,N-dimethylaminocinnamaldehyde binding to the binary complex formed between NADH and liver alcohol dehydrogenase. Absorbance changes (ΔA) calculated from the intercepts of the straight lines fitted to data such as those in Fig. 7

the amplitude thus determined with fixed concentrations of the aldehyde at pH 11.5. The reciprocal value of the intercept of the straight lines fitted to the data in Fig. 7 was taken to represent the equilibrium absorbance change (ΔA) due to complex formation at saturating concentration of NADH. The double-reciprocal plots in Fig. 8 illustrate that ΔA is hyperbolically dependent on the aldehyde concentration, and values of $K_{E \cdot NADH, ald}$ were calculated from such data as described by Dunn et al. [12].

The estimates of $K_{E \cdot NADH, ald}$ thus obtained at different pH between 10 and 12 are given in Fig. 9. They are fully consistent with the binding data reported for the pH range 6–10.5 by Dunn et al. [12, 15], and indicate that the magnitude of $K_{E \cdot NADH, ald}$ approaches a value of about 7 μ M at low pH. At high pH, there is a pronounced increase in magnitude of $K_{E \cdot NADH, ald}$ corresponding to an ionization step with a pK_a of 11.2 (± 0.2). The results in Fig. 9, therefore, provide direct evidence that aldehyde binding to the binary enzyme $\cdot$ NADH complex requires protonation of an ionizing group with a pK_a (11.2) agreeing with that (pK_{11}) affecting NADH dissociation from the binary complex.

DISCUSSION

Ionizing Groups Affecting Coenzyme Binding

We have concluded previously that all main effects of pH on coenzyme binding to liver alcohol dehydrogenase between pH 6 and 10 derive from ionization of the water molecule bound at the catalytic zinc ion of the enzyme subunit [7]. The present results will be discussed with the assumption that such is the case. The binding of NAD $^{+}$ below pH 10 is then known [3, 7] to be regulated by the protonation state of zinc-bound

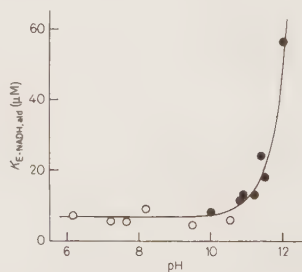


Fig. 9. Effect of pH on the equilibrium constant for trans-N,N-dimethylaminocinnamaldehyde dissociation from the ternary complex formed with NADH and liver alcohol dehydrogenase. Dissociation constants ($K_{E \cdot NADH, ald}$) now determined ($\bullet$) at 25 $^{\circ}$ C in 0.1 M ionic strength phosphate buffer, pH 10–12. Previously reported data ($\circ$) for the pH range 6–10.5 [12, 15] are included for comparison. The curve drawn corresponds to an ionization function with a pK_a of 11.2

water in free enzyme ($pK_f = 9.2$) and in the enzyme $\cdot$ NAD $^{+}$ complex ($pK_c = 7.6$) as expressed by Eqn (8), which provides a general relationship for the effect of an ionizing enzymic group on ligand binding to the enzyme. Complex formation with NADH exhibits an analogous pK_a -9.2-dependence attributable to ionization of zinc-bound water in free enzyme, the pK_f of this group being perturbed to a value above 10 on binding of the reduced coenzyme [3, 7]. According to Eqn (8), therefore, examination of the pH dependence of NADH binding above pH 10 should provide additional information about the actual pK_a of zinc-bound water in the enzyme $\cdot$ NADH complex. Such information is of interest for elucidation of the mechanisms by which coenzyme may affect the reactivity of zinc-bound ligands during the catalytic action of the enzyme.

The present investigation provides evidence that the binding of NADH at high pH is controlled by two

proton dissociation equilibria with pK_a values (10.4 and 11.2) within the examined pH range (10–12). The data in Fig. 3 demonstrate that the equilibrium with a pK_a of 10.4 also affects complex formation with NAD^+ . It follows from Eqn (8) and the results in Eqns (9) and (10) that the effect of the latter equilibrium on the coenzyme dissociation constants K_{E,NAD^-} and $K_{E,NADH}$ is such that it cannot reflect ionization of zinc-bound water; the origin of the pK_a -10.4-dependence of coenzyme binding will be briefly discussed below. It can be similarly inferred from Eqns (8–10) that the pK_a -11.2-dependence of NADH binding derives from an ionizing group in the enzyme $\cdot$ NADH complex. This group might be identical with zinc-bound water. If it is not, zinc-bound water must exhibit a pK_a value above 12 in the enzyme $\cdot$ NADH complex.

While the binding data *per se* provide no conclusive information about the identity of the pK_a -11.2-group, they do establish that this group exhibits a different pK_a in free enzyme (either 9.2 or 10.4, or a value outside the range 6–12) as well as in the enzyme $\cdot$ NAD^+ complex (either 7.6 or a value outside the range 6–12). It may then be noted that ligand-induced perturbations of the pK_a of an ionizing enzymic group reflect the effect of ionization of the group on the affinity of the enzyme for the ligand. NADH and NAD^+ have been shown to form structurally analogous complexes with liver alcohol dehydrogenase [16]. With respect to binding interactions of thermodynamic significance, the two coenzyme forms can be considered to be identical except for the difference in charge. If NADH and NAD^+ affect the pK_a of an ionizing enzymic group differently, therefore, this difference can be assumed to be of electrostatic origin, i.e. to reflect the coulombic interaction between the ionizing group and the positive charge on NAD^+ . This means that the ionizing group will have a lower pK_a in the enzyme $\cdot$ NAD^+ complex than in the enzyme $\cdot$ NADH complex.

It follows from these considerations that the pK_a -11.2-dependence of NADH binding can be anticipated to derive from an ionizing group which exhibits a pK_a of 7.6 (zinc-bound water) or lower than 6 (an alternative group) in the enzyme $\cdot$ NAD^+ complex. In other words, substitution of NADH by NAD^+ in the binary complex results in an electrostatic perturbation of the pK_a of the group by at least 3.6. As has been pointed out previously [3, 7], zinc-bound water appears to be the only ionizing group available which is located in sufficient proximity to the positive charge on enzyme-bound NAD^+ to undergo such a large electrostatic pK_a perturbation. The possibility that an alternative ionizing group could have its pK_a decreased by more than 5 through coulombic interaction with NAD^+ is rendered highly unlikely by reported crystallographic data for the enzyme [1]. For these reasons, we conclude that the observed pK_a -11.2-dependence of NADH binding to liver alcohol dehydrogenase most likely derives from

ionization of zinc-bound water in the enzyme $\cdot$ NADH complex.

The pK_a -10.4-dependence of coenzyme binding must derive from an ionizing group which exhibits a pK_a above 12 in the enzyme $\cdot$ coenzyme complexes, i.e. coenzyme binding is destabilized by a pK change of at least 1.6 on deprotonation of the group. This effect might reflect ionization of a group which participates directly in coenzyme binding (several candidates are available [1]), but might also be indirect and reflect pH-dependent destabilizing conformational changes of the enzyme. Since such conformational changes, in principle, could be induced by ionization of any amino acid residue with appropriate pK_a , it seems premature to speculate in more detail about the identity of the pK_a -10.4 group.

Aldehyde Binding to the Enzyme $\cdot$ NADH Complex

There is strong evidence indicating that alcohol and aldehyde substrates are directly coordinated to the catalytic zinc ion in the productive enzyme $\cdot$ coenzyme $\cdot$ substrate complexes formed during liver alcohol dehydrogenase catalysis [1, 2, 12, 16]. The binding of external ligands to the catalytic zinc ion appears to be prevented generally by ionization of the zinc-bound water molecule [7, 17]. In particular, alcohol association to the enzyme $\cdot$ NAD^+ complex has been shown to exhibit a pK_a -7.6-dependence consistent with this idea [5, 6]. Aldehyde binding to the enzyme $\cdot$ NADH complex, therefore, would be expected to be similarly dependent on the pK_a of zinc-bound water in the latter binary complex.

The results in Fig. 8 seem to establish the validity of that expectation. They demonstrate that binding of the chromophoric substrate *trans*-*N,N*-dimethylamino-cinnamaldehyde to the enzyme $\cdot$ NADH complex requires the protonated form of an ionizing group with a pK_a of 11.2. This observation provides, in a most crucial respect, confirmatory evidence that zinc-bound water exhibits a pK_a of 11.2 in the binary complex formed between liver alcohol dehydrogenase and NADH.

Effect of Ligand Field on the pK_a of Zinc-Bound Water

By analogy to the catalytic zinc ion in liver alcohol dehydrogenase [1], the active-site zinc ion in carboxypeptidase and carbonic anhydrase is bound by three protein ligands and coordinates a water molecule or hydroxyl ion as a fourth inner-sphere ligand [2]. Spectroscopic and kinetic evidence has been presented which indicates that the pK_a of zinc-bound water in carbonic anhydrase is about 7 [18]. This value has been considered as unreasonably low in comparison to the pK_a values observed for aquo ligands in six-coordinate zinc complexes [2]. A decrease in coordination number

of the metal, however, would be expected to increase the acidity of metal-bound water. This has been confirmed by measurement of the pK_a of aquo ligands in a number of six-coordinate and five-coordinate metal complexes. Wolley [19] concluded from such model studies that a pK_a of 7 is quite reasonable for zinc-bound water in carbonic anhydrase, where the four-coordinate zinc ion is bound by three histidyl residues [18].

The pK_a of zinc-bound water in carboxypeptidase appears to be close to 9 [20], i.e. about 2 higher than in carbonic anhydrase. Structural data for the two enzymes indicate that this difference in pK_a should be attributed primarily to the different inner-sphere ligand field at the active-site metal ion. The zinc ion in carboxypeptidase is bound by one glutamyl and two histidyl residues. The substitution of a neutral histidyl ligand by a negatively charged glutamyl ligand will reduce the coulombic force field between the zinc ion and zinc-bound water and raise the pK_a of the aquo ligand. To a first approximation, such an electrostatic pK_a perturbation may be assumed to show a linear free energy correlation with the number of negatively charged inner-sphere ligands. This means that zinc-bound water would be expected to exhibit a typical pK_a of about 11 in liver alcohol dehydrogenase, where the catalytic zinc ion is bound by one histidyl and two negatively charged cysteinyl residues [1].

Attention should then be drawn to our previous conclusion that the acidity of zinc-bound water in liver alcohol dehydrogenase is strongly affected also by outer-sphere ligand fields [7]. This conclusion was based on the observation that the pK_a of zinc-bound water is generally perturbed from 9.2 in free enzyme to values above 10 on complex formation with anionic reagents which combine to the pyrophosphate-binding part of the coenzyme-binding site. We attributed this pK_a perturbation to an indirect electrostatic interaction between the anionic reagent and the zinc-bound hydroxyl ion. Zinc-bound water was proposed to exhibit an atypically low pK_a in free enzyme due mainly to electrostatic stabilization of the zinc-bound hydroxyl ion by the positively charged guanidinium group of Arg-47. Such outer-sphere stabilization of the hydroxyl ion is prevented by the binding of NADH and related anionic reagents, which engage the guanidinium group of Arg-47 in salt-bridge formation at the pyrophosphate-binding subsite. According to this view, zinc-bound water should exhibit a more typical pK_a (less affected by strong outer-sphere ligand field contributions) in the binary enzyme · NADH complex. The pK_a value of 11.2 now assigned to zinc-bound water in the latter complex, therefore, is in excellent agreement with the value expected from the above discussion of the pK_a of metal-bound water in other zinc-containing enzymes. This lends credit to the present interpretation of the observed effects of high pH on coenzyme and

Table 2. Correlation between inner-sphere ligand field and the pK_a of zinc-bound water in enzymes containing a four-coordinate active site zinc ion

The pK_a value reported for liver alcohol dehydrogenase refers to the enzyme · NADH complex, in which outer-sphere ligand field effects on the acidity of zinc-bound water are of minor significance

Enzyme	Protein ligands	Total ligand charge	pK_a	Ref.
Carbonic anhydrase	His-93 His-95 His-117	0	7.0	[18]
Carboxypeptidase	His-142 His-146 Glu-166	-1	8.9	[20]
Liver alcohol dehydrogenase	His-67 Cys-47 Cys-174	-2	11.2	this work

aldehyde binding to liver alcohol dehydrogenase, as well as to our previous interpretation of the pK_a -9.2-dependence of coenzyme and anion binding to the enzyme [7].

Previous discussions of the pK_a of zinc-bound water in liver alcohol dehydrogenase have focused on the pK_a perturbations brought about by outer-sphere interactions with coenzyme and amino acid residues in the protein; such outer-sphere ligand field effects undoubtedly are of kinetic significance and mechanistic importance in the liver alcohol dehydrogenase system. The present results, however, draw attention to the fact that the pK_a of metal-bound water in zinc-containing enzymes must be determined primarily by inner-sphere ligand field effects. This is illustrated by the data summarized in Table 2, which indicate that the presence of negatively charged inner-sphere ligands drastically affects the pK_a of zinc-bound water. By analogy, then, inner-sphere ligand field effects would be expected to be of primary importance for regulation of the reactivity of zinc-bound substrates during enzyme turnover. This means that there should be explicit mechanistic reasons for the different modes of binding of the active-site zinc ion in the enzymes listed in Table 2. In a future publication we will consider some possible mechanistic explanations for the presence of two negatively charged inner-sphere protein ligands at the catalytic zinc ion of liver alcohol dehydrogenase.

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Synergism Between Coenzyme and Carboxylate Binding to Liver Alcohol Dehydrogenase

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1. The interaction of decanoate and benzoate with the substrate-binding site in liver alcohol dehydrogenase has been characterized by fluorimetric equilibrium binding studies, using auramine O as a reporter ligand.

2. The affinity of the enzyme for the carboxylates examined decreases about 50-fold on complex formation with NADH. The coenzyme-competitive inhibitors ADP-ribose and $\text{Pt}(\text{CN})_4^{2-}$ have a similar destabilizing effect on decanoate binding, indicating that the effect of NADH derives from its negatively charged pyrophosphate group.

3. Carboxylate binding to free enzyme requires the protonated form of an ionizing enzymic group with pK_a 9.2, while there is no corresponding effect of pH on binary complex formation with auramine O. Carboxylate binding to the enzyme · NADH complex exhibits no significant dependence on pH over the pH range 7–10.

4. These results, combined with previously reported data for decanoate binding to the enzyme · NAD^+ complex, provide clear evidence that carboxylates (in contrast to auramine O) are bound at the active-site zinc ion of the enzyme in a process regulated by the ionization state of zinc-bound water. The synergistic effect of NADH and NAD^+ on carboxylate binding can be qualitatively and quantitatively explained in terms of electrostatic interactions analogous to those proposed to account for the effect of coenzymes on the pK_a of zinc-bound water.

Coenzyme binding to liver alcohol dehydrogenase between pH 6 and 10 is strongly affected by two protonation equilibria involving, respectively, free enzyme (pK_a 9.2) and the binary enzyme · NAD^+ complex (pK_a 7.6) [1,2]. It appears well established that the pK_a -7.6-dependence of NAD^+ binding derives from ionization of the water molecule known to be bound at the active-site zinc ion of the enzyme subunit [1]. Recent investigations of the effect of pH on complex formation with anions and zinc-chelating reagents [3,4] led to the conclusion that ionization of zinc-bound water is likely to account also for the pK_a -9.2-dependence of NAD^+ and NADH association to free enzyme. This would imply that the pK_a of zinc-bound water is perturbed from 9.2 to a value above 10 on the binding of NADH [2,5], and evidence has been presented indicating that zinc-bound water actually exhibits a pK_a of 11.2 in the enzyme · NADH complex [6]. Andersson et al. [4] attributed this shift in pK_a to destabilization of the zinc-bound hydroxyl ion through (indirect) electrostatic interaction with the negatively charged pyrophosphate group of NADH. The corresponding destabilizing interaction in the enzyme · NAD^+ complex was envisaged to be counteracted by the stabilizing effect of the positively charged nicotinamide ring of the coenzyme. This was assumed to result in a net perturbation of the pK_a of zinc-bound water from 9.2 to 7.6 on the binding of NAD^+ .

There are important mechanistic implications of the proposal that differences in magnitude of the pK_a values regulating coenzyme binding to liver alcohol dehydrogenase reflect electrostatic interactions between coenzyme and the zinc-bound hydroxyl ion. For example, it follows that ligand binding to the active-site zinc ion (e.g. substrate binding resulting in formation of catalytically productive ternary

complexes) should be generally susceptible to coenzyme-induced electrostatic field effects of similar strength. To test the significance of such effects, we have examined the synergism between coenzyme binding and the binding of negatively charged substrate analogues (decanoate and benzoate) which are likely to combine to the active-site zinc ion on complex formation with the enzyme [1].

EXPERIMENTAL PROCEDURE

Materials

Crystalline horse liver alcohol dehydrogenase from Boehringer (Mannheim) was further purified as described by Bernhard et al. [7]. Enzyme concentrations were determined fluorimetrically by titration with NADH in the presence of isobutyramide [8] and are reported throughout as active-site concentrations.

The coenzyme NADH (Sigma Chemical Corp., grade III quality) was purified as described by Gurr et al. [9]. Other reagents were of highest available purity and used without further purification. Decanoic acid and benzoic acid were obtained from Merck (Darmstadt), $\text{K}_2\text{Pt}(\text{CN})_4$ and auramine O from Fluka AG (Buchs), and ADP-ribose from Sigma Chemical Corp. (St Louis, MO).

All experiments reported in the present paper were carried out at 25°C in 0.1 M ionic strength phosphate buffer solutions, prepared using water twice distilled from a quartz apparatus.

Methods

Equilibrium studies of complex formation at the substrate-binding site of liver alcohol dehydrogenase were performed as described by Sigman et al. [10]. Fluorescence changes asso-

Enzyme. Liver alcohol dehydrogenase or alcohol: NAD^+ oxidoreductase (EC 1.1.1.1).

ciated with auramine O binding to the enzyme were measured at 530 nm (excitation at 430 nm) using a Perkin-Elmer MPF-2A spectrofluorimeter. In typical experiments, a reaction solution (initially 2.5 ml) containing 0.25 μ M auramine O was titrated with 10- μ l aliquots of an about 100 μ M solution of enzyme in the presence of varied concentrations of decanoate or benzoate. The fluorescence (F) of the reaction solution was recorded after each addition of enzyme, apparent equilibrium constants $K_{app}(A)$ for auramine O dissociation being calculated from the regression function

$$\frac{1}{F} = \gamma \left(1 + \frac{K_{app}(A)}{c_E} \right) \quad (1)$$

where γ represents a proportionality constant and c_E stands for the total concentration of enzyme [10]. True equilibrium constants for auramine O ($K_{E,A}$) and the carboxylates examined ($K_{E,I}$) were obtained [10] from the relationship

$$K_{app}(A) = K_{E,A} \left(1 + \frac{[I]}{K_{E,I}} \right). \quad (2)$$

Determinations of $K_{E,A}$ were made separately in the absence of carboxylates [10].

Analogous relationships [10] were applied for evaluation of carboxylate binding data obtained in the presence of saturating concentrations of NADH (2 mM), ADP-ribose (5 mM), and $Pt(CN)_4^{2-}$ (20 mM). Control experiments were performed to establish that concentrations indicated were at least 95% saturating even at the highest carboxylate concentrations used in the latter binding studies.

Dissociation constant estimates reported represent the mean of at least three independent measurements, standard deviations of the estimates being less than 20% of the values given.

RESULTS

Effect of pH on Auramine O Binding

Equilibrium dissociation constants for the binding of auramine O to free liver alcohol dehydrogenase ($K_{E,A}$) and to the binary enzyme · NADH complex ($K_{E,R,A}$) were determined fluorimetrically [10] at different pH between 6 and 10. The results are given in Table 1 and establish that there is no significant effect of pH on the magnitude of $K_{E,A}$ and $K_{E,R,A}$. Dissociation constant estimates in Table 1 agree well with those reported previously for auramine O binding at pH 7 [10] and pH 7.4 [11].

Carboxylate Binding to Free Enzyme

The interaction of carboxylates with the substrate-binding site in the liver alcohol dehydrogenase subunit was examined fluorimetrically using auramine O as a reporter ligand. Control experiments confirmed previous reports that decanoate binds competitively with auramine O to the enzyme [10]. The equilibrium dissociation constant ($K_{E,I}$) for decanoate binding to free enzyme was determined as described by Sigman et al. [10] at different pH between 7 and 10. The results are given in Fig. 1 and show that $K_{E,I}$ increases most significantly in magnitude above pH 9. This indicates that binary-complex formation with decanoate requires protonation of an ionizing enzymic group exhibiting a pK_a value agreeing with that (9.2)

Table 1. Effect of pH on auramine O binding to liver alcohol dehydrogenase

Equilibrium dissociation constants determined fluorimetrically for auramine O binding to free enzyme ($K_{E,A}$) and to the enzyme · NADH complex ($K_{E,R,A}$) at 25 °C in 0.1 M ionic strength phosphate buffer

pH	$K_{E,A}$ μ M	$K_{E,R,A}$
6	8	4
7	12	4
8	10	5
9	13	5
10	11	4

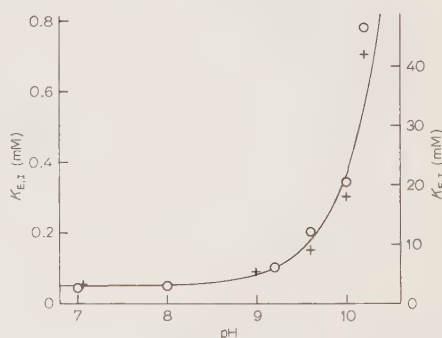


Fig. 1. Effect of pH on the affinity of liver alcohol dehydrogenase for carboxylates. Equilibrium constants $K_{E,I}$ for complex formation between enzyme and decanoate (O; left axis) or benzoate (+; right axis) determined fluorimetrically at 25 °C in 0.1 M ionic strength phosphate buffer using auramine O as a reporter ligand. The curve drawn corresponds to Eqn (3) with $pK_a = 9.2$.

previously attributed to ionization of zinc-bound water in free enzyme [3,4]. Confirmatively, the ionization function

$$K_{E,I} = K_{E,I}^* \left(1 + \frac{K_a}{[H^+]} \right) \quad (3)$$

could be well fitted to the data in Fig. 1 with the assumption that $pK_a = 9.2$, which gave the best-fit estimate $K_{E,I}^* = 50 (\pm 7) \mu$ M.

The pH dependence of the equilibrium dissociation constant $K_{E,I}$ for benzoate binding to free enzyme was similarly determined (Fig. 1) and found to conform to Eqn (3) with $pK_a = 9.2$ and $K_{E,I}^* = 3.0 (\pm 0.6)$ mM.

Carboxylate Binding to the Enzyme · NADH Complex

The effect of NADH on the apparent equilibrium dissociation constant [$K_{app}(I)$] for complex formation between liver alcohol dehydrogenase and decanoate was examined by the same fluorimetric method. The typical results in Fig. 2, referring to data obtained at pH 7, show that the magnitude of $K_{app}(I)$ increases towards a limiting value of 2.4 mM with increasing concentrations of NADH. Experimental data used for calculation of $K_{app}(I)$ at 2 mM NADH are given in Fig. 3. They illustrate that the linear dependence on decanoate concentration of the apparent affinity of the enzyme for auramine O persists at saturating concentrations of coenzyme. These ob-

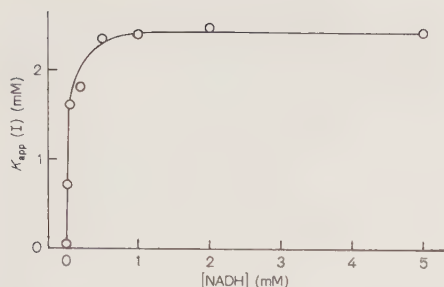


Fig. 2. Effect of NADH on the affinity of liver alcohol dehydrogenase for decanoate. Apparent equilibrium dissociation constants $K_{app}(I)$ for complex formation between enzyme and decanoate determined fluorimetrically at 25°C in 0.1 M ionic strength phosphate buffer containing varied concentrations of NADH

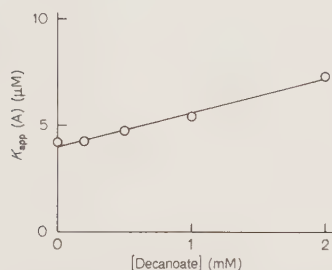


Fig. 3. Decanoate binding to the binary complex formed between liver alcohol dehydrogenase and NADH. Apparent equilibrium dissociation constants $K_{app}(A)$ for complex formation between enzyme and auramine O determined fluorimetrically at 25°C in 0.1 M ionic strength phosphate buffer containing 2 mM NADH and varied concentrations of decanoate

servations are inconsistent with a competitive binding of NADH and decanoate, but provide clear evidence that a (comparatively weak) ternary enzyme · NADH · decanoate complex is formed at high enough concentrations of the ligands. The limiting value of $K_{app}(I)$, which can be taken as a measure of the equilibrium constant $K_{ER,1}$ for decanoate dissociation from the ternary complex, was found to remain essentially unaffected by pH over the pH range 7–10 (Table 2).

The equilibrium dissociation constant for benzoate binding to the enzyme · NADH complex was similarly determined at pH 7, which gave $K_{ER,1} = 150 (\pm 30)$ mM. Hence one obtains a value of 50 for the quotient $K_{ER,1}/K_E^1$ with benzoate as the ligand I. Calculation of the corresponding datum for decanoate binding at pH 7 gave $K_{ER,1}/K_E^1 = 48$. It may be concluded that NADH binding decreases the affinity of the enzyme for the carboxylates examined by approximately the same factor of about 50.

Decanoate Binding to the Enzyme · ADP-ribose and Enzyme · $Pt(CN)_4^-$ Complexes

The effect on decanoate binding of the coenzyme-competitive [1,12] inhibitors ADP-ribose and $Pt(CN)_4^-$ was examined at pH 7 in experiments analogous to those described for NADH in the preceding section. Both coenzyme analogues were found to form ternary complexes with enzyme and

Table 2. Effect of pH on decanoate binding to the binary complex formed between liver alcohol dehydrogenase and NADH. Equilibrium constants $K_{ER,1}$ for decanoate dissociation calculated from binding data such as those in Fig. 3

pH	$K_{ER,1}$ mM	pH	$K_{ER,1}$ mM
7	2.4	9	3.0
8	2.2	10	3.9

decanoate, the affinity of the enzyme for decanoate decreasing by a factor of 25 or 13 on the binding of ADP-ribose or $Pt(CN)_4^-$, respectively. These observations indicate that the interaction between enzyme and decanoate is generally weakened on the binding of ligands which utilize the anion-binding subsite [1] of the coenzyme-binding site. It may be concluded that the destabilizing effect of NADH on carboxylate binding is likely to be mainly of electrostatic origin and to derive from the negatively charged pyrophosphate group of the coenzyme.

DISCUSSION

Binding Sites for Carboxylates

The substrate-binding region in the liver alcohol dehydrogenase subunit consists of a hydrophobic barrel leading from solution to the active-site zinc ion [1]. The dye auramine O is known to interact specifically with this hydrophobic region to give a highly fluorescent adduct [11]. Coenzyme binding results in a slight enhancement of the affinity of enzyme for auramine O (cf. Table I), while substrates and substrate analogues displace the dye competitively [10]. Complex formation at the substrate-binding site, therefore, can be conveniently monitored fluorimetrically using auramine O as a reporter ligand. The present application of this site-specific method for examination of ligand binding to liver alcohol dehydrogenase establishes that decanoate and benzoate (and carboxylates in general, by inference) combine to the substrate-binding region in free enzyme, as well as in the enzyme · NADH complex.

This does not preclude the existence of additional sites for interaction of carboxylates with the enzyme. In particular, it cannot be excluded that certain carboxylic acids may interact strongly with the coenzyme-binding region in free enzyme, as suggested by binding data obtained crystallographically and in solution with salicylic acid derivatives [13]. Complex formation with the latter compounds, however, appears to be dependent on specific binding interactions (hydrogen bonding of the carboxyl group of Asp-223 by the phenolic hydroxyl group of salicylate) which cannot be at hand with the carboxylates now examined. Benzoate, therefore, would be expected to exhibit much lower affinity than salicylate for the coenzyme-binding site. This means, according to data in Fig. 1 and those reported for complex formation with salicylate [13], that benzoate is likely to be more firmly bound as a substrate analogue than as a coenzyme analogue.

The same certainly applies for complex formation with decanoate. The present estimate of the equilibrium constant for decanoate binding to free enzyme at pH 7 ($K_{E,1} = 45$ μM, referring to complex formation at the substrate-binding site) agrees with that reported by Theorell et al. [14]. Since the

latter estimate was obtained from measurements which did not discriminate between interactions at the substrate-binding and the coenzyme-binding sites, it may be concluded that the complex formed at the former site must be the tightest one. Consequently, decanoate and benzoate binding in competition with coenzyme cannot possibly be firm enough to cause any significant displacement of NADH in binding studies such as those reported in Fig. 3. The saturation behaviour with increasing NADH concentration of $K_{app}(I)$ for carboxylate binding (Fig. 2) undoubtedly reflects saturation of the enzyme with NADH, and data in Table 2 can be safely assumed to represent equilibrium constants for decanoate binding to the enzyme · NADH complex.

Effect of pH on the Binding of Carboxylates and Auramine O

The active-site zinc ion in liver alcohol dehydrogenase is bound by three protein ligands, a tetrahedral coordination being completed by a water molecule projecting into the hydrophobic region where substrates and auramine O bind [1]. Coordination of enzyme-bound auramine O by the zinc ion would have to take place through interaction with the π -electrons of the nitrogen of one of the *p*-dimethylamino substituents of this diphenylmethane derivative. Reported structural data for the enzyme [1] indicate that formation of such a coordination bond can be anticipated to be sterically hindered. Recent fluorescence polarization measurements seem to confirm that auramine O does not combine to the zinc ion on complex formation with the enzyme [15].

On the other hand, it appears well established that alcohol and aldehyde substrates are directly bound to the active-site zinc ion in the productive ternary complexes formed during catalysis [1, 16–18]. A carboxylate ligand, brought from solution to the substrate-binding region through hydrophobic interactions, would not be expected to show a lower affinity for the metal than do alcohol and aldehyde ligands. The mere observation that carboxylates combine to the substrate-binding site on complex formation with the enzyme can be taken as a strong indication that they actually become ligated to the active-site zinc ion.

More direct evidence in this direction is provided by the observed pH dependence of carboxylate binding, which agrees with that established for complex formation with ligands known to combine to the active-site zinc ion. Decanoate binding to the enzyme · NAD⁺ complex requires protonation of an enzymic group with a pK_a of 7.6 [19], as does the binding of alcohols [18] and zinc-chelating reagents [3]. Binary-complex formation with carboxylates (Fig. 1), as well as with zinc-chelating reagents [3, 4], is analogously regulated by an ionizing group showing a pK_a of 9.2 in free enzyme, while there is no significant effect of pH (below pH 10) on the binding of aldehydes [16, 18], zinc-chelating reagents [4], or decanoate (Table 2) to the enzyme · NADH complex. This pH dependence pattern has been proposed to reflect that complex formation involving ligand binding to the active-site zinc ion is prevented by ionization of the zinc-bound water molecule [1, 6, 18]. The present results are obviously consistent with that idea.

As has been previously discussed in detail [2], there seems to be no reasonable alternative explanation for the observed pK_a -7.6-dependence of ligand binding to the enzyme · NAD⁺ complex. Proposals that the pK_a -9.2 group in free enzyme can be identified as zinc-bound water [3, 4], however, have been based implicitly on the assumption that the observed effect of pH on binary-complex formation with zinc-chelating

Table 3. Effect of coenzymes on proton and decanoate binding to liver alcohol dehydrogenase

pK_a values attributed to deprotonation of zinc-bound water in different enzymes species [2, 6] compared with pK values for decanoate dissociation from the complex formed with the binding protonation state of these species

Species	H ⁺		C ₉ H ₁₉ COO ⁻		reference
	pK_a	ΔpK_a	pK	ΔpK	
Enzyme	9.2		4.3		this work
Enzyme · NAD ⁺	7.6	-1.6	5.9	+1.6	[14, 19]
Enzyme · NADH	11.2	+2.0	2.6	-1.7	this work

reagents reflects changes in the coordinative properties of the active-site zinc ion. This assumption seems reasonable, but its validity has not previously been verified. Ionization of the pK_a -9.2 group prevents complex formation with a variety of ligands [4], including coenzymes, coenzyme fragments, anions, zinc-chelating reagents, and (according to the present results) carboxylates. The effect of pH on complex formation with zinc-chelating reagents, therefore, might not be specific for ligand binding to the active-site zinc ion, but could reflect a pH-dependent conformational change with drastic general effects on the binding of ligands which interact with the active-site region of the enzyme. Such a conformational change, in principle, could be triggered by deprotonation of any ionizing enzymic group.

Attention should then be drawn to the data in Table 1, which establish that there is no significant effect of pH on auramine O binding to free enzyme over the pH range 6–10. This observation provides direct evidence that ionization of the pK_a -9.2 group has no general effect on complex formation at the substrate-binding site, but affects only the binding of substrate analogues which combine to the active-site zinc ion. Consequently, it seems justified to assume that the coordinative properties of the zinc ion are drastically affected by ionization of the pK_a -9.2 group. The present results, therefore, lend crucial support to the previous conclusion [3, 4] that this group can be identified as zinc-bound water.

Effect of Coenzymes on Carboxylate Binding

In a systematic study of the binding of saturated fatty acids at pH 7, Winer and Theorell found that the affinity of liver alcohol dehydrogenase for carboxylates increases most significantly on complex formation with NAD⁺ [20]. The present results indicate that complex formation with NADH, as well as with ADP-ribose and Pt(CN)₅³⁻, leads to a corresponding general decrease in affinity of the enzyme for carboxylates at pH values where the enzyme is mainly in the binding protonation state. These observations are obviously qualitatively consistent with the idea that the synergism between carboxylate and coenzyme binding derives from electrostatic interactions analogous to those proposed to regulate the acidity of the ionizing group identified as zinc-bound water [4].

Table 3 compares the pK_a values attributed to deprotonation of zinc-bound water in different enzyme species [2, 6] with pK values determined previously [14] or in the present work for decanoate dissociation from the complex formed with the binding protonation state of these species. Examination of the data shows that the stabilizing effect of NAD⁺ on

decanoate binding agrees (within experimental precision) with the destabilizing effect of NAD^+ on proton binding to the ionizing enzymic group. NADH has the converse effect of stabilizing binding of the positively charged proton to approximately the same extent as it destabilizes binding of the decanoate anion. These observations seem to establish that the coenzyme-induced effects on complex formation with the two oppositely charged ligands do derive from analogous electrostatic interactions, the effect of the positively charged nicotinamide ring of NAD^+ greatly exceeding that of the negatively charged pyrophosphate group of the coenzyme. It can be inferred from the absolute and relative strength of these interactions (as indicated by the observed pK and pK_a shifts) that the proton and the negative charge on decanoate must be bound in close proximity to the nicotinamide ring of enzyme-bound NAD^+ . This and other arguments leading to the conclusion that the ionizing enzymic group can be identified as zinc-bound water have been previously discussed in detail [4,6]. It may suffice to point out here that data in Table 3 reinforce that conclusion, as well as the conclusion that carboxylates combine to the active-site zinc ion on complex formation with the enzyme. The qualitative and quantitative results in Table 3 can be well understood in terms of a coenzyme-induced electrostatic stabilization/destabilization of, respectively, the zinc-bound hydroxyl ion and the zinc-bound carboxylate ion. Considering the structural and kinetic information available from previous studies of the liver alcohol dehydrogenase system [1,3,4], there seems to be no reasonable alternative explanation for the observations now made.

Summing up the above discussion, the present investigation provides a plausible explanation for the observed effects of pH and coenzyme binding on complex formation between liver alcohol dehydrogenase and carboxylate ligands. More importantly, it confirms the validity of previous assignments of kinetically determined pK_a values in Table 3 to ionization of zinc-bound water [1,3,4,6] and establishes the general significance of electrostatic field effects of coenzymes on zinc-bound ligands. Zinc-bound alcohols, therefore, must be assumed to be subjected to analogous field effects, resulting in pK_a perturbations similar to those of water, in the productive ternary complexes formed with enzyme and NAD^+ . This lends credence to the mechanism of enzyme action proposed by Kvassman and Pettersson [18], according to

which alcoholate ion formation at the ternary-complex level represents an obligatory reaction step facilitating hydride transfer from alcohol substrates to NAD^+ .

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Electrostatic field effects of coenzymes on ligand binding to catalytic zinc in liver alcohol dehydrogenase

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1. The synergism between coenzyme and anion binding to liver alcohol dehydrogenase has been examined by equilibrium measurements and transient-state kinetic methods to characterize electrostatic interactions of coenzymes with ligands which are bound to the catalytic zinc ion of the enzyme subunit.

2. Inorganic anions typically exhibit an at least 200-fold higher affinity for the general anion-binding site than for catalytic zinc on complex formation with free enzyme. Acetate and SCN^- interact more strongly with catalytic zinc in the enzyme $\cdot \text{NAD}^+$ complex than with the general anion-binding site in free enzyme. CN^- shows no significant affinity for the general anion-binding site, but combines to catalytic zinc in the absence as well as the presence of coenzymes.

3. Coordination of CN^- to catalytic zinc weakens the binding of NADH by a factor of 50, and tightens the binding of NAD^+ to approximately the same extent through interactions which do not include any contributions from covalent adduct formation between CN^- and NAD^+ . These observations provide unambiguous information about the magnitude of electrostatic field effects of coenzymes on anion (e.g. hydroxyl ion) binding to catalytic zinc. They lead to the important inference that coenzyme binding must be strongly affected by ionization of zinc-bound water irrespective of the actual acidity of the latter group.

4. It is concluded on such grounds that the much debated pH dependence of coenzyme binding to liver alcohol dehydrogenase must derive from ionization of zinc-bound water. The assumption that such is not the case leads to the inference that there is no detectable effect of ionization of zinc-bound water on coenzyme binding over the pH range 6–12, a possibility which is definitely excluded by the present results.

The effect of pH on the catalytic activity of liver alcohol dehydrogenase has been extensively studied and intensively discussed [1, 2]. Such discussions have focused on the identity and mechanistic significance of the ionizing groups which control coenzyme and substrate binding to the enzyme. We have attributed the observed pH dependence of coenzyme binding to electrostatic interactions reflecting ionization of the water molecule which is bound at the catalytic zinc ion of the enzyme subunit [3]. Charged groups on NADH and NAD^+ were assumed to interact strongly enough with a zinc-bound hydroxyl ion to account for the pK_a values assigned to zinc-bound water in different enzyme species [4]. This would require that ionization of zinc-bound water increases (decreases) the affinity of the enzyme for NAD^+ (NADH) by a factor of 40 (100).

Evidence suggesting that zinc-bound anions may give rise to electrostatic field effects of that order of magnitude has been obtained by examination of the synergism between coenzyme binding and the binding of negatively charged substrate analogues such as decanoate and benzoate [5, 6]. Complex formation between enzyme and the latter carboxylates was found to result in a 40-fold stabilization of NAD^+ binding and a slightly larger destabilization of the binding of NADH [6]. If these effects can be assumed to derive from charge interactions between coenzyme and a zinc-bound carboxylate anion, then it would seem sufficiently well established that (and how) coenzyme binding is controlled by the ionization state of zinc-bound

water. This would lend credence also to the proposal that NAD^+ -induced ionization of zinc-bound alcohols represents a key step in the catalytic oxidation of alcohol substrates [7].

Unfortunately, however, the observed synergism between coenzyme and carboxylate binding cannot be unequivocally attributed to charge interactions. Decanoate and benzoate are bound mainly through hydrophobic interactions at the substrate-binding site, and there is reason to believe that complex formation at this site may affect coenzyme binding independently of ligand charge [8]. The observed quantitative analogy between equilibrium constants for carboxylate and proton dissociation from different enzyme species, therefore, might be fortuitous or might reflect interactions which should not be related primarily to complex formation at the catalytic zinc ion. Complementary information is obviously required to draw any firm conclusions about the strength and catalytic significance of charge interactions between coenzymes and zinc-bound anions.

The present investigation was undertaken to obtain such information. Data now reported seem to establish that cyanide (in contrast to a large number of inorganic anions previously examined [9]) combines to catalytic zinc on complex formation with liver alcohol dehydrogenase. We have, therefore, characterized the synergism between coenzyme and cyanide binding to the enzyme by equilibrium measurements and transient-state kinetic methods. The results provide conclusive evidence that electrostatic interactions between coenzymes and zinc-bound ligands are of crucial importance for the binding properties, and hence likely also for the catalytic reactivity, of liver alcohol dehydrogenase.

Enzyme. Liver alcohol dehydrogenase or alcohol:NAD oxidoreductase (EC 1.1.1.1).

EXPERIMENTAL PROCEDURE

Materials

Crystalline horse liver alcohol dehydrogenase from Boehringer (Mannheim) was further purified as described by Bernhard et al. [10], except that 50 mM phosphate buffer was used for elution of the enzyme in the column chromatographic purification step. Enzyme concentrations were determined fluorimetrically by titration with NADH in the presence of isobutyramide [11] and are reported throughout as active-site concentrations.

NADH and NAD^+ (Sigma Chemical Corp., grade III quality) were purified as described by Gurr et al. [12]. Other reagents were of highest available quality and used without further purification. Cyanide solutions were prepared from the sodium salt and adjusted to appropriate pH with phosphoric acid. Concentrations of CN^- were calculated using a $\text{p}K_a$ value of 9.2 for ionization of HCN [13].

Methods

All experiments reported in the present paper were carried out at 25 °C in 5–20 mM phosphate buffer, pH 7.7 (unless otherwise stated). A Durrum D-110 stopped-flow spectrometer, equipped as detailed previously [14], was used for the study of rapid reactions. Other measurements were made with a Shimadzu UV-240 spectrophotometer.

Equilibrium data for complex formation between liver alcohol dehydrogenase (about 30 μM) and 2,2'-bipyridine (0.2–3 mM) were determined by the spectrophotometric method of Sigman [15]. The kinetics of bipyridine binding to the enzyme were examined as described by Andersson et al. [3]. Complex formation between enzyme (1–10 μM) and NAD^+ (0.5–200 μM) was monitored photometrically at 275 nm by stopped-flow techniques. Spectrophotometric equilibrium measurements of NADH binding were made at 355 nm with 40 μM enzyme. The kinetics of NADH binding were studied fluorimetrically by stopped-flow techniques, using about 1 μM enzyme. The excitation wavelength was 330 nm, and a Corning 0-52 filter was used to avoid registration of light emitted at wavelengths below 340 nm.

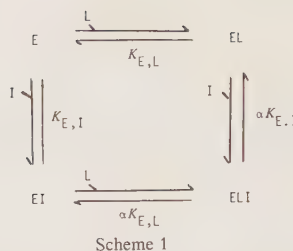
Effects of cyanide and other anions on the above reactions were examined after a 2–5-min preincubation of the enzyme with the respective anion. No effects of cyanide attributable to extraction of catalytic zinc from the enzyme could be detected. Absorbance changes associated with the binding of bipyridine agreed with those expected from the enzyme concentration used whether or not reactions were performed in the presence of cyanide (cf. Fig. 1).

Data evaluation

Absorbance changes (ΔA) observed on complex formation between liver alcohol dehydrogenase and the reporter ligands (L) bipyridine, NADH, and NAD^+ , were evaluated by regression analysis using the relationship

$$1A = \frac{\Delta \epsilon \cdot c_E}{1 + \frac{K_{\text{app}}}{[L]}} \quad (1)$$

where c_E represents the total concentration of enzyme, and $\Delta \epsilon$ denotes the difference absorption coefficient for binding of the respective ligand. When total concentrations of ligand (c_L) did



not greatly exceed c_E , mutual depletion effects were accounted for by combining Eqn (1) with the stoichiometric relationship

$$[L] = \frac{1}{2} [c_L - c_E - K_{\text{app}} + \sqrt{(c_L - c_E - K_{\text{app}})^2 + 4c_L K_{\text{app}}}] \quad (2)$$

Apparent reporter ligand dissociation constants (K_{app}) determined in the presence of anions (I) were interpreted in view of Scheme 1, which allows for the formation of ternary enzyme · ligand · anion complexes with a cooperativity factor α . Assuming that anion binding contributes negligibly to the absorbance changes observed, Scheme 1 prescribes that

$$K_{\text{app}} = K_{E,L} \frac{1 + \frac{[I]}{K_{E,I}}}{1 + \frac{[I]}{\alpha K_{E,I}}} \quad (3)$$

where $K_{E,L}$ and $K_{E,I}$ denote equilibrium constants for reporter ligand and anion dissociation from the respective binary complex. When anions are bound in competition with the reporter ligand Eqn (3) reduces to

$$K_{\text{app}} = K_{E,L} \left(1 + \frac{[I]}{K_{E,I}} \right) \quad (4)$$

Apparent first-order rate constants (r) for the transients governing coenzyme (L) binding were calculated by regression analysis [16]. Estimates thus obtained were evaluated in terms of the linear relationship

$$r = k_{\text{on}}[L] + k_{\text{off}} \quad (5)$$

Effects of anions on the apparent rate constants for coenzyme association (k_{on}) and dissociation (k_{off}) were interpreted with the assumption that anions equilibrate rapidly with the enzyme.

RESULTS

Cyanide binding to free enzyme

Complex formation between liver alcohol dehydrogenase and cyanide was examined at different pH by standard spectrophotometric equilibrium methods, using the zinc-chelating reagent 2,2'-bipyridine as a reporter ligand [15]. The competitive effect of cyanide on bipyridine binding is illustrated by the typical results in Fig. 1. Equilibrium dissociation constants for the enzyme · cyanide complex were calculated from such data by application of Eqns (1) and (4), and the results are given in Table 1. Dissociation constants relating to the total concentration of cyanide are strongly pH-dependent, whereas those relating to the cyanide ion concentration remain essentially constant ($K_{E,\text{CN}^-} = 12 \mu\text{M}$) between pH 6 and 9. This observation provides clear evidence that the competitive effects of cyanide are attributable to the CN^- species and not to HCN. Evaluation of results obtained at pH 6.1 showed that CN^- must be bound at least 10000 times more tightly than HCN.

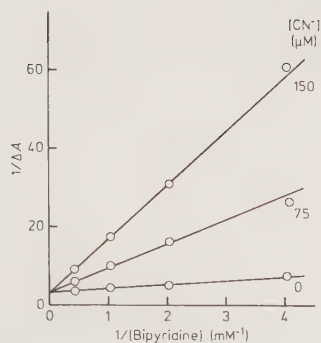


Fig. 1. Effect of cyanide on the equilibrium binding of 2,2'-bipyridine to liver alcohol dehydrogenase. Absorbance changes (ΔA) recorded at 312 nm and 25 °C on titration of the enzyme (30 μM) with bipyridine in 5–20 mM phosphate buffer, pH 7.7, containing varied concentrations of CN^- .

Table 1. Cyanide binding to liver alcohol dehydrogenase

Dissociation equilibrium constants referring to the total concentration of cyanide and the concentration of CN^- , respectively, determined at 25 °C from the effect of cyanide on bipyridine binding to the enzyme in 5–20 mM phosphate buffer. Standard deviations of estimates are 10–20% of the values given

pH	Dissociation constants	
	$K_{E,\text{cyanide}}$	$K_{E,\text{CN}}$
	μM	
6.1	15 000	12
7.0	1 700	11
7.7	400	12
8.5	80	14
9.2	50	27
10.0	160	140

This means that the $\text{p}K_a$ value for ionization of HCN decreases by more than 4 when the acid is ligated to the enzyme.

Complex formation between enzyme and a variety of ligands (including anions [17]) is known to require protonation of an ionizing enzymic group (presumably zinc-bound water) with a $\text{p}K_a$ value close to 9.2. The increase in magnitude of the estimates of K_{E,CN^-} obtained above pH 9 (Table 1) indicates that the binding of CN^- is similarly dependent on the ionization state of the $\text{p}K_a$ -9.2 group.

Rate constants for binary-complex formation between enzyme and CN^- were determined at pH 7.7 by transient-state kinetic displacement methods [19]. Bipyridine binding to the enzyme- CN^- complex was found to be rate-limited by an apparent first-order process with a rate constant tending towards 25 s^{-1} with increasing bipyridine concentrations. According to the kinetic theory for displacement reactions and reported data for bipyridine binding [3], the latter value can be taken to provide a comparatively precise estimate of the rate constant for CN^- dissociation from the enzyme. This estimate corresponds (Table 1) to a CN^- association rate constant of approximately $2\text{ s}^{-1}\mu\text{M}^{-1}$, sufficiently high to justify the rapid-equilibrium assumptions required to arrive at Eqn (5).

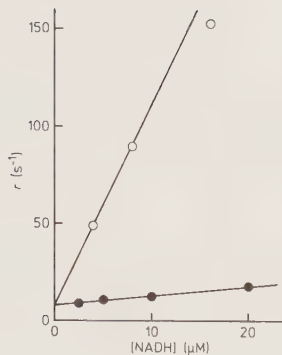


Fig. 2. Effect of cyanide on the kinetics of NADH binding to liver alcohol dehydrogenase. Apparent first-order rate constants (r) determined at 25 °C in 10 mM phosphate buffer, pH 7.7, for the transient governing NADH binding to the enzyme (1.2 μM) in the absence (○) or presence (●) of 300 μM CN^- .

Complex formation between enzyme and CN^- was found not to be associated with any detectable absorption changes in the 300–800-nm region. This justifies application of Eqns (3) and (4) for evaluation of the synergism between cyanide and coenzyme binding.

Synergism between cyanide and NADH binding

Fig. 2 shows the effect of 300 μM CN^- on the transient-state kinetics of NADH binding at pH 7.7. As previously observed with other anions examined [9], CN^- appears to act as a competitive inhibitor of NADH binding over the tested range of coenzyme concentrations. The estimate of K_{E,CN^-} calculated from data in Fig. 2 agreed well with that (12 μM) determined using bipyridine as reporter ligand. Similar results were obtained in measurements performed at pH 6 and 7.

Data in Fig. 2 do not distinguish between a competitive and a strongly (negatively) cooperative binding of NADH and CN^- . Evidence that the two ligands actually form a weak ternary complex with the enzyme was obtained by spectrophotometric equilibrium measurements, which showed that apparent NADH dissociation constants (K_{app}) increased towards a saturation value of about 20 μM with increasing concentrations of CN^- . This is illustrated in Fig. 3 by example of the NADH titration curve obtained in the presence of 12 mM CN^- . Assuming that $K_{E,\text{NADH}} = 0.5\mu\text{M}$ [19] and $K_{E,\text{CN}^-} = 12\mu\text{M}$ (Table 1), a K_{app} value of about 500 μM would be expected for NADH binding in competition with CN^- (dashed curve in Fig. 3). A fit of Eqns (1) and (2) to data in Fig. 3 gave $K_{app} = 23 (\pm 3)\mu\text{M}$, however, which corresponds to a cooperativity factor $\alpha = 48$ in Eqn (3). Similar analysis of data obtained with 1.2 mM CN^- gave $K_{app} = 17\mu\text{M}$ and $\alpha = 53$. It may be concluded from these results that NADH and CN^- form an enzymic ternary complex which is synergistically destabilized by a factor of approximately 50.

Adduct formation between cyanide and NAD^+

CN^- is known to form an adduct with NAD^+ in a slow second-order process [20, 21]. This process is accelerated (but not catalyzed) by liver alcohol dehydrogenase in the sense that the addition of enzyme to solutions containing NAD^+ and

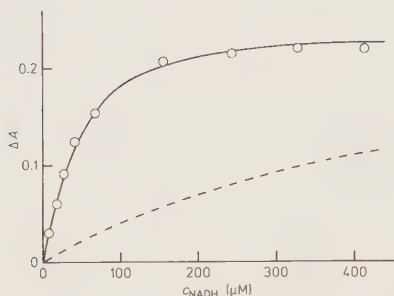


Fig. 3. Effect of cyanide on the equilibrium binding of NADH to liver alcohol dehydrogenase. Absorbance changes (ΔA) recorded at 355 nm on titration of the enzyme (40 μM) with NADH at pH 7.7 in the presence of 12 mM CN^- . Experimental points conform to Eqns (1) and (2) with $K_{\text{app}} = 23 \mu\text{M}$. The dashed curve corresponds to NADH binding in competition with CN^- .

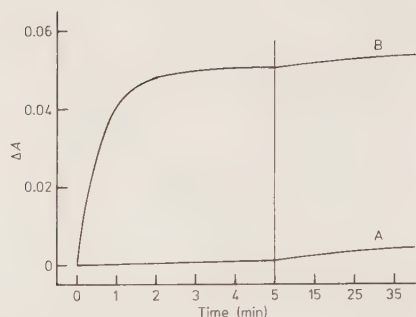


Fig. 4. Kinetics of adduct formation between NAD^+ and cyanide. Absorbance changes (ΔA) recorded at 310 nm for the reaction of 50 μM NAD^+ with 50 μM CN^- in the absence (curve A) or presence (curve B) of 10 μM liver alcohol dehydrogenase. Measurements were performed at 25 °C in 10 mM phosphate buffer, pH 7.7.

CN^- leads to rapid formation of an inactive enzyme·adduct complex with maximum absorption at 310 nm [22]. Fig. 4 shows that such complex formation occurs in a first-order transient at a rate which greatly exceeds that of non-enzymic adduct formation at the reactant concentrations used. The rate constant for this transient (0.027 s^{-1}) was found to remain essentially unaffected by variation of the concentrations of NAD^+ (20–500 μM) and CN^- (20–1200 μM).

These observations provide evidence that the enzyme-bound adduct is formed via an exceptionally tight enzyme· NAD^+ · CN^- complex. To avoid interferences from non-enzymic or enzymic reactions of CN^- with NAD^+ , kinetic and equilibrium data for the latter complex were determined exclusively by stopped-flow techniques.

Synergism between cyanide and NAD^+ binding

Fig. 5 shows the variation with coenzyme concentration of apparent first-order rate constants (r) for the transient governing NAD^+ binding at pH 7.7 in the presence of 690 μM CN^- . Data conform well to Eqn (5) with $k_{\text{on}} = 0.70 (\pm 0.05) \text{ s}^{-1} \mu\text{M}^{-1}$ and $k_{\text{off}} < 2 \text{ s}^{-1}$. The corresponding parameter values for complex formation with NAD^+ in the absence of CN^- are approximately $k_{\text{on}} = 1 \text{ s}^{-1} \mu\text{M}^{-1}$ and $k_{\text{off}} = 70 \text{ s}^{-1}$ [19]. It may be concluded from results in Fig. 5, therefore, that CN^- binding drastically increases the affinity of the enzyme for NAD^+ by decreasing the rate of coenzyme dissociation.

The kinetics of NAD^+ binding to the enzyme· CN^- complex were similarly examined at pH 10, which gave a k_{on} value ($0.6 \text{ s}^{-1} \mu\text{M}^{-1}$) differing insignificantly from that obtained at pH 7.7. This indicates that the pK_a 9.2 dependence of coenzyme association to free enzyme [19] is eliminated by CN^- binding to the enzyme.

Amplitudes of the transient governing NAD^+ binding to the enzyme· CN^- complex provide a direct measure of the absorbance changes associated with formation of the enzyme· NAD^+ · CN^- complex. Such equilibrium data are given in Fig. 6 for the reaction at pH 7.7 in the presence of 690 μM CN^- . Application of Eqns (1) and (2) for regression analysis of the data gave $K_{\text{app}} = 1.4 (\pm 0.2) \mu\text{M}$ as a best-fit estimate of the apparent NAD^+ dissociation constant. This corresponds to a cooperativity factor $\alpha = 0.019$ for NAD^+ and

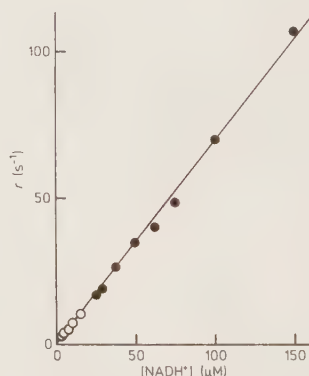


Fig. 5. Effect of cyanide on the kinetics of NAD^+ binding to liver alcohol dehydrogenase. Apparent first-order rate constants (r) determined at 25 °C for the transient governing NAD^+ binding to the enzyme (10 μM , $\circ$; 1 μM , $\bullet$) in 11 mM phosphate buffer, pH 7.7, containing 690 μM CN^- .

CN^- binding, calculated from Eqn (3) with the assumption that $K_{\text{E} \cdot \text{NAD}^+} = 70 \mu\text{M}$ [19] and $K_{\text{E} \cdot \text{CN}^-} = 12 \mu\text{M}$ (Table 1).

Anion binding to the enzyme· NAD^+ complex

Fig. 7 shows the coenzyme concentration dependence of apparent first-order rate constants for the transient governing NAD^+ binding at pH 7.7 in the presence of SCN^- . The observed effects on the k_{on} value for NAD^+ binding agree well with those expected from reported equilibrium data for the coenzyme-competitive binding of this anion [9]. The decreased magnitude of the k_{off} value (70 s^{-1} in the absence of SCN^- [19]) cannot be explained in terms of such anion binding, but indicates that a ternary enzyme· NAD^+ · SCN^- complex forms with an anion dissociation equilibrium constant of about 0.1 mM. Acetate was analogously found to form an enzyme· NAD^+ ·anion complex with an anion dissociation constant close to 2 mM.

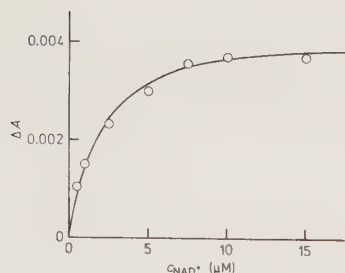


Fig. 6. Effect of cyanide on the equilibrium binding of NAD^+ to liver alcohol dehydrogenase. Absorbance changes (ΔA) recorded by stopped-flow techniques at 275 nm on reacting the enzyme ($1.0 \mu\text{M}$) with NAD^+ in 11 mM phosphate buffer, pH 7.7, containing $690 \mu\text{M}$ CN^- . The curve drawn was calculated from Eqns (1) and (2) for $K_{\text{app}} = 1.4 \mu\text{M}$

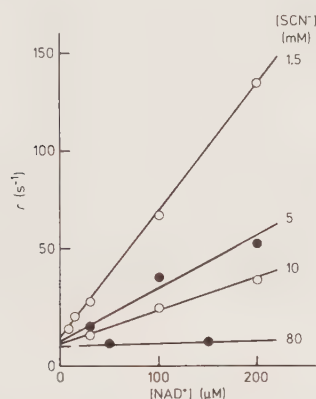


Fig. 7. Effect of thiocyanate on the kinetics of NAD^+ binding to liver alcohol dehydrogenase. Apparent first-order rate constants (r) determined at 275 nm for the transient governing NAD^+ binding to the enzyme ($10 \mu\text{M}$) in about 10 mM phosphate buffer, pH 7.7, containing varied concentrations of SCN^-

No similar effects on the apparent rate of NAD^+ dissociation were detected in the presence of about 80% saturating concentrations [9] of other coenzyme-competitive anions tested: Br^- , HPO_4^{2-} , $\text{Pt}(\text{CN})_4^{2-}$, SO_4^{2-} . Effects of decanoate on the kinetics of NAD^+ binding were closely analogous to those of CN^- .

DISCUSSION

Binding site for cyanide

The coenzyme-binding region of the liver alcohol dehydrogenase subunit contains a hydrophobic subsite at which the guanidinium group of Arg-47 provides a positive charge for salt-bridge formation with the pyrophosphate group of bound NADH or NAD^+ [1]. It has been well documented that this arginyl subsite functions as a general binding site for inorganic anions and carboxylates such as formate and acetate [23]. Anion binding at this site prevents complex formation between

enzyme and coenzyme competitively, but has no negative effect on bipyridine binding to catalytic zinc [19].

The present results establish that cyanide is bound as CN^- to liver alcohol dehydrogenase in strict competition with bipyridine. Furthermore, ternary enzyme · coenzyme · CN^- complexes form with both NAD^+ and NADH. These observations provide clear evidence that CN^- interacts with a site distinct from the general anion-binding site. Considering the exceptional metal-coordinative properties of the ligand and the fact that bipyridine binding is competitively affected, there seems to be no doubt that CN^- combines to catalytic zinc on complex formation with the enzyme. Spectroscopic data consistent with metal coordination of CN^- have been reported for the interaction of this ligand with enzyme in which catalytic zinc was substituted by nickel [24] or copper [25].

The interaction of CN^- with the general anion-binding site appears to be weak compared to the interaction with catalytic zinc. This can be inferred from the absence of a pronounced effect of CN^- on the apparent on-velocity constant for NAD^+ binding to the enzyme (Fig. 5). The latter rate parameter should be competitively affected by complex formation at the general anion-binding site [9], and data in Fig. 7 confirm that such effects are at hand with a coenzyme-competitive ligand such as SCN^- .

Synergism between coenzyme and anion binding

Equilibrium measurements now performed (Fig. 3 and 6) establish that there is a pronounced synergistic cooperativity in the binding of coenzymes and CN^- to liver alcohol dehydrogenase. Coordination of CN^- to catalytic zinc weakens the binding of NADH by a factor of 50, and tightens the binding of NAD^+ to approximately the same extent through interactions which do not include any contributions from covalent adduct formation. Since a zinc-bound cyanide ion is unlikely to interfere sterically with coenzyme binding [1], the observed cooperativity effects can be assumed to derive almost exclusively from electrostatic interactions. According to previously advanced ideas [3], charge interactions of zinc-bound CN^- with the coenzyme pyrophosphate group (probably mediated by the side chain of Arg-47) are likely to provide a major contribution to the observed destabilization of NADH binding. Stabilization of the binding of NAD^+ is certainly achieved through charge interaction of CN^- with the coenzyme nicotinamide ring, which is bound in close proximity to catalytic zinc [1].

Cooperativity factors now determined for coenzyme and CN^- binding agree closely in magnitude with those reported previously for the synergism between coenzyme and decanoate binding [6]. This confirms that the observed synergistic effects are predominantly of electrostatic origin, and suggests that anion binding to catalytic zinc is generally about two orders of magnitude tighter in the presence of enzyme-bound NAD^+ than in the absence of coenzyme. Consequently, complex formation between enzyme and NAD^+ might render anion binding to catalytic zinc detectably tight also with ligands which combine preferentially to the Arg-47 subsite in their interaction with free enzyme. The present attempts to demonstrate such ligation to zinc by kinetic methods (through effects on the k_{off} value for NAD^+ binding) failed with most anions tested. This indicates that anions in general interact at least 200 times more strongly with the Arg-47 subsite than with catalytic zinc in the absence of coenzymes.

The latter results establish that the nicotinamide ring of NAD^+ cannot substitute for Arg-47 as the cationic constituent

of a site with strong general anion-binding properties. The observation that comparatively tight enzyme·NAD⁺·anion complexes form with SCN⁻ (Fig. 7) and acetate, therefore, can be best understood in terms of anion binding to catalytic zinc. The former ligand (analogously with CN⁻) shows a high tendency for metal coordination in general, and ligation of acetate to catalytic zinc (analogously with the binding of decanoate [6]) may be stabilized through interaction of the ligand with the substrate-binding site. Judging from the observed effects on the apparent NAD⁺ dissociation rate, SCN⁻ and acetate appear to be bound 15–30-times more tightly to the enzyme·NAD⁺ complex than to free enzyme. Consequently, there is no reason to doubt that the two anions show a higher affinity for the Arg-47 subsite than for catalytic zinc in their interaction with free enzyme. Ligation to zinc, however, might be of primary importance for the inhibitory effects of acetate and SCN⁻ on the catalytic activity of the enzyme.

Depending on the length of the side-chain, ligation to catalytic zinc may (decanoate [6]) or may not (formate, acetate [9]) predominate in the binary enzymic complexes formed with fatty acids. Binding data obtained in our laboratory indicate that the metal site is preferred by propionate and higher homologues.

Ionization of zinc-bound water

CN⁻ binding to catalytic zinc and ionization of zinc-bound water are closely analogous processes, the latter being thermodynamically equivalent to hydroxyl ion binding. These processes, therefore, should be energetically affected in the same direction and to approximately the same extent by the electrostatic field changes associated with coenzyme binding. Table 2 illustrates that there is such a correspondence and quantitative agreement between the observed effects of coenzymes on equilibrium constants for anion dissociation from catalytic zinc and proton dissociation from the ionizing enzymic group which accounts for the pH dependence of coenzyme binding between pH 6 and 10. Data in Table 2 are in obvious consistence with previously reported evidence identifying this ionizing group as zinc-bound water [1–4, 18]. The present results, in fact, would seem to exclude any alternative explanation for the main effects of pH on coenzyme binding on the basis of the following arguments.

With respect to interactions of the nature and strength now discussed, NAD⁺ and NADH may be considered to differ exclusively as concerns the positive charge on the nicotinamide ring of oxidized coenzyme. Differences in the pH dependence of NAD⁺ and NADH binding, therefore, can be attributed to electrostatic field effects of this positive charge on proton binding to the enzyme. Since such effects will favour deprotonation of the enzyme irrespective of the nature of the ionizing group, the pH dependence of the quotient $K_{E,NAD^+}/K_{E,NADH}$ must include superimposed contributions from all ionizing groups which interact strongly enough with the positive charge on NAD⁺. The magnitude of the contribution provided by ionization of zinc-bound water can be estimated from the observed difference (3.4) between $p\alpha$ values for CN⁻ binding to catalytic zinc in the binary enzyme·coenzyme complexes (Table 2). It follows from this datum that the binding of NADH and/or NAD⁺ must be strongly affected by ionization of zinc-bound water irrespective of the actual acidity of the latter group. Due to the field effect of the positive charge on NAD⁺, the pK_a value for zinc-bound water will be drastically (about 3.4) lower in the enzyme·NAD⁺ complex than in the

Table 2. Effect of coenzymes on proton and anion binding to liver alcohol dehydrogenase

pK_a values attributed to deprotonation of zinc-bound water in different enzyme species [3, 4] compared with cooperativity factors (α) for the binding of coenzymes and decanoate [6] or cyanide

Species	H ⁺		C ₉ H ₁₉ COO ⁻	CN ⁻
	pK_a	ΔpK_a	$p\alpha$	$p\alpha$
Enzyme	9.2			
Enzyme·NAD ⁺	7.6	-1.6	+1.6	+1.7
Enzyme·NADH	11.2	+2.0	-1.7	-1.7

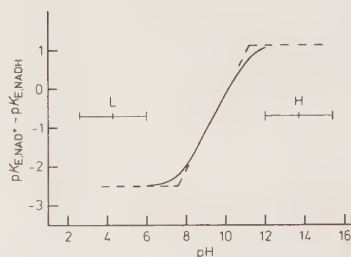


Fig. 8. Electrostatic field effect of the positively charged nicotinamide ring of NAD⁺ on proton binding to liver alcohol dehydrogenase. pH dependence of the quotient between equilibrium constants for dissociation of the binary enzymic complexes formed with oxidized (K_{E,NAD^+}) and reduced ($K_{E,NADH}$) coenzyme [4, 19]. If the observed effect of pH is assumed not to derive from ionization of zinc-bound water, then unreasonably high (range H or higher) or low (range L or lower) pK_a values must be assigned to zinc-bound water in enzyme species listed in Table 2

enzyme·NADH complex, and the quotient $K_{E,NAD^+}/K_{E,NADH}$ must exhibit a most pronounced variation with pH in the corresponding pH region.

Fig. 8 shows the pH dependence of $K_{E,NAD^+}/K_{E,NADH}$ over the pH range 6–12, as characterized by previously reported binding studies [4, 19]. The latter results provide the important inference that interactions between the positive charge on NAD⁺ and ionizing enzymic groups with pK_a values between 6 and 12 are of detectable strength only in one instance. The observed effect of pH on $K_{E,NAD^+}/K_{E,NADH}$ derives exclusively from ionization of the enzymic group considered in Table 2, i.e. the one showing of pK_a of 7.6 (11.2) in the enzyme·NAD⁺ (enzyme·NADH) complex. The effect is seen in the pH region where zinc-bound water would be expected to ionize [4, 26], and the strength of the charge interaction ($\Delta pK_a = 3.6$) agrees excellently with that predicted for zinc-bound water by anion binding in Table 2 ($\Delta p\alpha = 3.3–3.4$).

Attribution of the pH effect in Fig. 8 to an ionizing group distinct from zinc-bound water, however, would lead to the conclusion that zinc-bound water does not contribute detectably to the pH dependence of $K_{E,NAD^+}/K_{E,NADH}$ over the pH range examined. This would require that pK_a values for zinc-bound water in enzyme species listed in Table 2 either range from 2.5–6 (or lower) or from 12–15.5 (or higher), alternatives which are completely inconsistent with present knowledge about the coordination chemistry of zinc [26, 27]. The assumption that zinc-bound water ionizes with a pK_a slightly

above 12 in the enzyme · NAD⁺ complex would imply that the enzyme · NADH complex contains a zinc-bound water molecule which is more basic than free water, and such cannot possibly be the case; the decrease in pK_a by more than 4 now documented for HCN in its interaction with free enzyme confirms the obvious expectation that zinc coordination of water must result in a considerable increase in acidity of the ligand. The possibility that pK_a values for zinc-bound water fall within the range 2.5–6 (or lower) seems equally unrealistic, and can be definitely excluded on kinetic grounds. Such low pK_a values would render apparent rates of water dissociation from the metal too low to be reconciled with observed rates of ligand (e.g. bipyridine) binding to catalytic zinc in free enzyme and the enzyme · NAD⁺ complex [7, 18, 28]. The present results, therefore, can be taken to provide conclusive evidence identifying the ionizing group considered in Table 2 as zinc-bound water.

According to that inference, coenzyme binding to enzyme lacking catalytic zinc would be expected to show no significant dependence on pH over the range 7–10, unless the freed cysteinyl zinc ligands remain unoxidized after removal of the metal ion. In the latter case, however, coenzyme binding should be affected electrostatically by ionization of groups with approximately the same pK_a and location as zinc-bound water, i.e. would be expected to show a pH dependence similar to that observed with native enzyme. The latter expectation has been recently confirmed with enzyme from which the active-site metal ion was removed under anaerobic conditions [29]. The lack of effect of pH on coenzyme binding to zinc-free enzyme prepared under aerobic conditions was demonstrated by Coleman et al. in 1972 [30].

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V



Anion Binding to Liver Alcohol Dehydrogenase

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1. Complex formation at the general anion-binding site of the liver alcohol dehydrogenase subunit has been characterized by transient-state kinetic methods, using NADH as a reporter ligand. Equilibrium dissociation constants for anion binding at the site are reported. They conform basically to the lyotropic series of affinity order, with exceptionally tight binding of sulphate. The particular specificity for sulphate might be a general characteristic of anion-binding enzymic arginyl sites.

2. Anionic species of phosphate and pyrophosphate buffer solutions do not interact significantly with the general anion-binding site over the pH range 8–10. At lower pH, phosphate binding becomes significant due to complex formation with the monovalent H_2PO_4^- species. The latter interaction corresponds to a dissociation constant of about 60 mM, indicating that phosphate binding is comparatively weak also at low pH.

3. It is concluded that previously reported pH dependence data for coenzyme binding to liver alcohol dehydrogenase cannot be much affected by coenzyme-competitive effects of buffer anion binding. Kinetic parameter estimates now determined for NADH binding in weakly buffered solutions agree within experimental precision with those obtained previously from measurements made in buffer solutions of 0.1 M ionic strength.

The pyrophosphate-binding subsite of the coenzyme-binding region in liver alcohol dehydrogenase appears to function as a general site for complex formation with anionic ligands [1,2]. Dahl et al. [3] recently drew attention to the interaction of buffer anions with this site. They found by Cys-46 alkylation experiments that phosphate binding at the site (which has previously been thought to be of minor significance [4–6]) corresponds to an apparent dissociation constant as low as 20 mM at pH 7. Since most studies of liver alcohol dehydrogenase have been performed in phosphate buffer of at least 0.1 M ionic strength, Dahl et al. concluded that published kinetic and equilibrium data for coenzyme binding may be strongly influenced by unaccounted effects of coenzyme-competitive buffer anion binding.

This conclusion has some alarming implications as concerns reported pH dependence data for the coenzyme binding process. There is a notable lack of knowledge about the ligand specificity of the pyrophosphate-binding subsite with respect to inorganic anions in general, and anionic species of standard buffer systems in particular. We cannot even tell whether the interaction with phosphate observed at pH 7 [3,4] is attributable mainly to H_2PO_4^- , HPO_4^{2-} or PO_4^{3-} binding. This means that all pH dependence studies of coenzyme binding to liver alcohol dehydrogenase may have been performed in reaction solutions containing varied concentrations of different competitive anions. Data obtained under such conditions could be drastically biased in several respects. For example, pH effects due to buffer protonation equilibria might overlap and even mask effects due to ionizing groups on the enzyme. It cannot be excluded, therefore, that inferences drawn from reported effects of pH on coenzyme binding may be incorrect in essential respects. Considering that such inferences have formed a basis for several fundamental proposals relating to the mechanism of liver alcohol dehydrogenase catalysis, it seems of outmost importance to eliminate

the interpretational uncertainty introduced by the observations and conclusion of Dahl et al. [3].

To this end, we have carried out a systematic study of anion binding to liver alcohol dehydrogenase by examining the effect of various neutral salts and buffer systems on the transient-state kinetics of complex formation between enzyme and NADH. Results now reported characterize the anion specificity of the pyrophosphate-binding subsite, and hence provide some inferences regarding the properties of a structurally well-defined general anion-binding site. This makes it possible also to estimate the magnitude and discuss the significance of previously unaccounted effects of buffer anion binding.

EXPERIMENTAL PROCEDURE

Materials

Crystalline horse liver alcohol dehydrogenase from Boehringer (Mannheim) was further purified as described by Bernhard et al. [7], except that 50 mM phosphate buffer was used for elution of the enzyme in the column chromatographic purification step. Enzyme concentrations were determined fluorimetrically by titration with NADH in the presence of isobutyramide [8] and are reported throughout as active-site concentrations.

The coenzyme NADH (Sigma Chemical Corp., grade III quality) was purified as described by Gurr et al. [9]. Other reagents were of highest available quality and used without further purification.

Methods

All experiments reported in the present paper were carried out at 25°C in 1 mM (unless otherwise stated) phosphate buffer solutions of appropriate pH between 6.6 and 10. The transient-state kinetics of NADH binding to liver alcohol dehydrogenase were examined by stop-flow techniques, using

Enzyme. Liver alcohol dehydrogenase or alcohol:NAD⁺ oxidoreductase (EC 1.1.1.1).

the equipment and methods detailed previously [10]. The enzyme (about 2 μM) was reacted with varied concentrations of NADH (5–150 μM) in the presence of 25 μM *trans*-*N,N*-dimethylaminocinnamaldehyde and selected concentrations of anions tested. Reactions were initiated by mixing enzyme with other reactants. Absorbance changes due to NADH binding (and subsequent rapid formation of a chromophoric ternary complex [10]) were monitored at 464 nm, and were invariably found to be governed by a single-exponential transient. The corresponding apparent first-order rate constant for the binding process was estimated by statistical regression methods [11]. Effects of pyrophosphate and carbonate buffer anions on NADH binding were examined over the pH range 8–10 in reaction solutions containing 10 mM phosphate.

The effect of anions on the apparent dissociation equilibrium constant for complex formation between liver alcohol dehydrogenase and bipyridine was examined spectrophotometrically by bipyridine titration experiments analogous to those described by Sigman [12]. Measurements were made at pH 8 in 10 mM phosphate buffer using a Beckman Acta CIII spectrophotometer.

RESULTS

Transient-State Kinetic Model

Let us consider the reaction mechanism in Scheme 1, according to which the binding of NADH or a coenzyme-competitive anion (A) to liver alcohol dehydrogenase (E) requires the protonated form of an ionizing group on the enzyme [10,13]. Assuming that protons and anions equilibrate much more rapidly than coenzyme with the enzyme, Scheme 1 prescribes that absorbance changes associated with NADH binding will be governed by a single exponential transient when coenzyme is present in large excess to the enzyme [14]. The apparent first-order rate constant (r) for this transient is given by

$$r = k_{\text{off}} + k_{\text{on}} [\text{NADH}] \quad (1)$$

where

$$k_{\text{off}} = k_{-1} \quad (2)$$

$$k_{\text{on}} = \frac{k_1}{1 + \frac{[\text{A}]}{K_A} + \frac{K_9}{[\text{H}^+]}} \quad (3)$$

K_A and K_9 denote the equilibrium constant for, respectively, anion and proton dissociation from the enzyme. Eqn (3) may be written as

$$k_{\text{on}} = \frac{k_{1,\text{app}}}{1 + \frac{K_{9,\text{app}}}{[\text{H}^+]}} \quad (4)$$

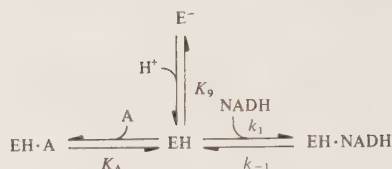
where

$$k_{1,\text{app}} = \frac{k_1}{1 + \frac{[\text{A}]}{K_A}} \quad (5)$$

$$K_{9,\text{app}} = \frac{K_9}{1 + \frac{[\text{A}]}{K_A}} \quad (6)$$

NADH Binding in the Absence of Inhibitory Amounts of Anions

The apparent first-order rate constant (r) for the transient governing NADH binding to liver alcohol dehydrogenase [10]



Scheme 1. Kinetic scheme used for interpretation of observed effects of anions and pH on NADH binding to liver alcohol dehydrogenase. A denotes a coenzyme-competitive anion and EH the binding protonation state of the enzyme. Anions and protons are assumed to equilibrate rapidly with enzyme. K_A and K_9 stand for the corresponding equilibrium dissociation constants

was determined for a series of coenzyme concentrations in 1 mM phosphate buffer solutions of appropriate pH between 7 and 10. Apparent NADH association (k_{on}) and dissociation (k_{off}) rate constants were calculated by fitting Eqn (1) to the data obtained at each fixed pH. Estimates of k_{off} were found to remain constant (about 5 s^{-1}) within experimental precision over the examined pH range. The pH dependence of k_{on} is indicated in Fig. 1 (curve A) and conforms to Eqn (4) with $k_{1,\text{app}} = 13 (\pm 2) \mu\text{M}^{-1} \text{s}^{-1}$ and $\text{p}K_{9,\text{app}} = 9.0 (\pm 0.2)$. As will be shown below, these parameter values can be interpreted in terms of Eqns (5) and (6) with the assumption that no anion binding is at hand ($[\text{A}] = 0$), i.e. they may be considered to provide direct estimates of the parameters k_1 and $\text{p}K_9$, respectively.

Effect of Neutral Salt Anions on NADH Binding

Experiments described in the preceding section were repeated using reactant solutions containing different fixed concentrations of Na_2SO_4 . The typical results in Fig. 2, referring to measurements made at pH 7, show that the linear dependence of the transient rate constant r on NADH concentration persists in the presence of inhibitory concentrations of the salt. Control experiments performed with K_2SO_4 , $(\text{NH}_4)_2\text{SO}_4$, or MgSO_4 gave results indistinguishable from those obtained with the corresponding concentration of Na_2SO_4 , indicating that effects observed are attributable to the anionic component of the salts.

Interpreted in terms of Eqn (1), the results in Fig. 2 indicate that SO_4^{2-} affects mainly the apparent association rate constant (k_{on}) for NADH binding to the enzyme. The replot in Fig. 3 shows that the reciprocal value of k_{on} is linearly dependent on the anion concentration. These observations are consistent with Eqns (2) and (3) and provide direct evidence that SO_4^{2-} acts as a strictly competitive inhibitor of NADH binding. A fit of Eqn (3) to the data in Fig. 2 with the assumption that $[\text{H}^+] \gg K_9$ gave $k_1 = 13 (\pm 2) \mu\text{M}^{-1} \text{s}^{-1}$ and $K_A = 8.8 (\pm 1.6) \text{mM}$.

Data obtained for SO_4^{2-} inhibition of coenzyme binding at higher pH were similarly found to be consistent with Eqns (1–3) and an inhibitor dissociation constant close to 8 mM. We prefer to illustrate this by example of the observed effects of pH on k_{on} for NADH association in the presence of 40 mM SO_4^{2-} (Fig. 1, curve B). A fit of Eqn (4) to the latter results gave $k_{1,\text{app}} = 2.4 (\pm 0.3) \mu\text{M}^{-1} \text{s}^{-1}$ and $\text{p}K_{9,\text{app}} = 9.7 (\pm 0.2)$, which agrees excellently with the theoretical values calculated from Eqns (5) and (6) using $K_A = 8.8 \text{mM}$ and the estimates of k_1 and $\text{p}K_9$ determined in the preceding section.

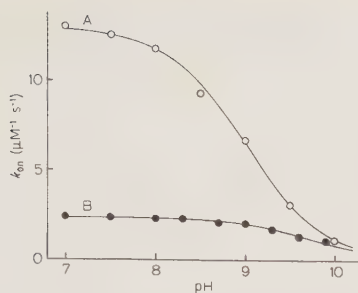


Fig. 1. Effect of pH on NADH binding to liver alcohol dehydrogenase. Apparent rate constants (k_{on}) for NADH association to the enzyme determined at 25°C in 1 mM phosphate buffer containing no added anions (curve A) or 40 mM sulphate ion (curve B)

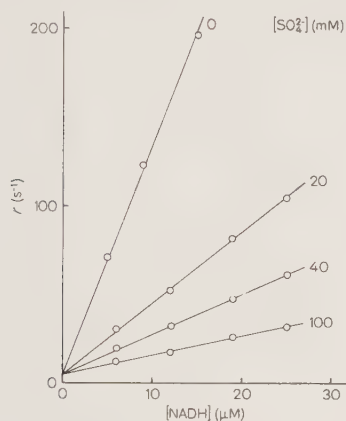


Fig. 2. Effect of sulphate ion on NADH binding to liver alcohol dehydrogenase. Apparent first-order rate constants (r) for the transient governing complex formation between enzyme (about 2 μ M) and varied concentrations of NADH, determined at 25°C in 1 mM phosphate buffer (pH 7.0) containing different fixed concentrations of sulphate ion

Analogous transient-state kinetic inhibition studies were carried out at pH 7 for a series of monovalent anions whose acid counterparts exhibit pK_a values well below 6. Anion effects observed were invariably found to be strictly competitive with NADH association, i.e. to conform to the kinetic pattern prescribed by Eqns (1–3) for $[H^+] \gg K_9$. Estimates of the respective anion dissociation equilibrium constants K_A were calculated from data analogous to those in Fig. 2 and 3, and the results are given in Table 1.

Control experiments performed at pH 9 and 10 established that the inhibitory action of two representative anions (NO_3^- and I^-) was consistent with Eqn (3) for $pK_9 = 9.0$, and gave estimates of K_A agreeing well with those obtained for the respective anion at pH 7.

Effect of Buffer Anions on NADH Binding

Effects on coenzyme binding of anions which form part of buffer systems were analogously examined. The kinetics of NADH binding between pH 8 and 10, as determined in solutions containing 1 mM phosphate, were not detectably

Table 1. Anion binding to liver alcohol dehydrogenase

Dissociation equilibrium constants (K_A) determined from the effect of anions on the NADH association rate at 25°C in 1 mM phosphate buffer, pH 7.0. Standard deviations of estimates are 10–15% of the values given

Anion	K_A
	mM
F^-	> 200
CH_3COO^-	60
Cl^-	42
$HCOO^-$	37
Br^-	19
SO_3^{2-}	8.8
NO_3^-	8.4
I^-	5.3
ICH_2COO^-	4.8
ClO_4^-	3.5
SCN^-	1.4
$Pt(CN)_4^{2-}$	0.04

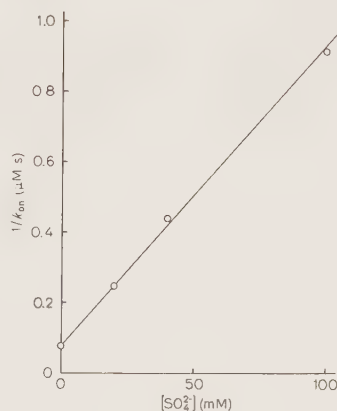


Fig. 3. Dependence on sulphate ion concentration of k_{on} values calculated from data in Fig. 2. The line drawn corresponds to Eqn (3) with $k_1 = 13 \text{ s}^{-1} \mu\text{M}^{-1}$, $K_A = 8.8 \text{ mM}$, and $K_9 \ll [H^+]$

affected when the phosphate buffer concentration was raised to 100 mM. Considering the experimental precision, this observation indicates that the corresponding apparent K_A values for phosphate binding certainly exceed 200 mM. Below pH 8, phosphate inhibited NADH binding competitively (Fig. 4). Apparent K_A values, relating to the total concentration of phosphate, were calculated from Eqn (3) using $pK_9 = 9.0$ and the results obtained at different pH are given in Table 2.

The absence of detectable effects of phosphate buffer above pH 8 provides direct evidence that effects observed at lower pH are attributable exclusively to complex formation between enzyme and the monovalent $H_2PO_4^-$ species. The validity of this conclusion is confirmed by the satisfactory constancy of corrected K_A values in Table 2, which refer to the actual concentration of $H_2PO_4^-$ at different pH [15] and are indicative of complex formation with a $H_2PO_4^-$ dissociation constant of about 60 mM.

It could be similarly inferred from measurements performed over the pH range 8–10 that carbonate buffer interferes competitively with NADH binding due to complex

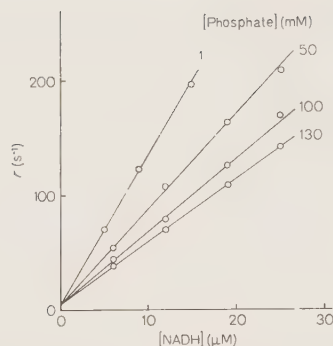


Fig. 4. Effect of phosphate buffer on NADH binding to liver alcohol dehydrogenase. Apparent first-order rate constants (r) for the transient governing complex formation between enzyme (about $2 \mu\text{M}$) and varied concentrations of NADH, determined at 25°C in buffer solutions (pH 7.0) containing different fixed concentrations of phosphate

Table 2. Phosphate binding to liver alcohol dehydrogenase

Dissociation equilibrium constants (K_A) determined from the dependence on phosphate buffer concentration of the NADH association rate at 25°C . Uncorrected values refer to the total concentration of phosphate, corrected ones to the concentration of H_2PO_4^- . Standard deviations of estimates are 10–15% of the values given

pH	K_A	
	uncorrected	corrected
	mM	
6.6	75	60
7.0	90	55
7.3	150	66
8.0	> 200	—
9.0	> 200	—
10.0	> 200	—

formation between enzyme and HCO_3^- with a (corrected) K_A value of $110 (\pm 20)$ mM. Pyrophosphate had no detectable effect on coenzyme binding between pH 8 and 10 when tested at a total buffer concentration of 50 mM.

K_A values reported above (including those in Table 1) were calculated with the assumption that the presence of 1 mM (10 mM) phosphate buffer contributes negligibly to the observed anion effects on NADH binding over the pH range 7–10 (8–10). Results in Table 2 establish the validity of that assumption.

Effect of Anions on Bipyridine Binding

The effect of some representative anions on bipyridine binding to the catalytic zinc ion in the liver alcohol dehydrogenase subunit [1] was studied at pH 8 by standard spectrophotometric equilibrium methods [12]. All anions examined [HCO_3^- , CH_3COO^- , SO_4^{2-} , I^- , $\text{Pt}(\text{CN})_4^{2-}$, SCN^-] were found to increase the apparent affinity of the enzyme for bipyridine by a factor varying from 1.2 (HCO_3^- , CH_3COO^-) to 1.8 (SO_4^{2-}) when tested at a concentration fivefold higher than the respective K_A value in Table 1. This appears to exclude the possibility that observed coenzyme-competitive effects

(Table 1) of the latter anions reflect ligation to the catalytic zinc ion.

DISCUSSION

Properties of the General Anion-Binding Site

The pyrophosphate-binding subsite of the coenzyme-binding region in the liver alcohol dehydrogenase subunit can be characterized as a shallow hydrophobic pocket at which the guanidinium group of Arg-47 provides a positive charge for strong electrostatic interactions [1,2]. There is crystallographic, kinetic, and spectral evidence indicating that anionic ligands in general are bound at this site [2,16–18]. Such complex formation, including NADH binding, has been reported to require the protonated form of an ionizing enzymic group (probably zinc-bound water) and to perturb the pK_a of this group from about 9 to a value well above 10 [10,13,19]. As will be discussed in the following section, there is no reason to doubt the validity of these conclusions on the basis of unaccounted effects of buffer binding. The simplified reaction mechanism in Scheme 1, therefore, should be adequate for description of the kinetic effects of anion binding on complex formation between enzyme and NADH at pH values up to 10.

This is confirmed by the present results. Salt effects on NADH binding now reported exhibit no direct correlation with ionic strength or cation concentrations, but undoubtedly derive from complex formation between enzyme and anions present in the reaction solutions. Over the examined ranges of pH and ligand concentrations, this interaction appears to be strictly competitive with coenzyme binding for all anions tested, such that Eqns (1–3) apply within experimental precision. The control experiments now performed establish that observed effects on NADH binding of representative anions are attributable to an interaction which is certainly not competitive with bipyridine binding to the catalytic zinc ion. It seems reasonable to assume, therefore, that all anions in Table 1 combine to the pyrophosphate-binding subsite of the coenzyme-binding region.

Anion binding at hydrophobic enzymic sites containing a histidyl residue as the main cationic constituent has been previously examined and found to conform to the well-known lyotropic (Hofmeister) series of affinity order [20,21]. Data in Table 1 establish that essentially the same anion binding order applies for complex formation at the pyrophosphate-binding arginyl subsite in liver alcohol dehydrogenase. This confirms the supposition of Fraústo da Silva and Williams [21] that anion binding according to the lyotropic series is primarily related to the hydrophobic environment of the interacting positive charge on the enzyme, and less dependent on the nature of the enzymic group which carries the charge.

The thermodynamic factors giving rise to the lyotropic series of anion binding are comparatively well understood [21] and will not be considered here. It may suffice to point out that affinity data in Table 1 exhibit only one notable deviation from this series. The highly hydrated divalent sulphate ion would be expected to be more loosely bound than any of the monovalent anions in Table 1. The actual affinity of sulphate for the pyrophosphate-binding subsite, however, is comparable to that of lowly hydrated anions such as bromide and nitrate. This indicates that the subsite exhibits a particular specificity for sulphate.

Anion binding to glucose-6-phosphate dehydrogenase has been reported to conform to a similar affinity order with extra-

ordinarily tight binding of sulphate [22]. Several enzymes containing arginyl sites are actually known to interact exceptionally strongly with sulphate [23]. The possibility cannot be excluded that the particular specificity for sulphate of the general anion-binding site in liver alcohol dehydrogenase resides in the binding properties of a hydrophobic arginyl site as opposed to a histidinyll or lysyl site. The anion binding properties of hydrophobic cationic sites may not be entirely independent of the nature of the group which carries the positive charge.

Effect of Buffer Anions

Eqs (5) and (6) prescribe that the presence of a coenzyme-competitive anion will cause an apparent decrease of the rate of coenzyme association to liver alcohol dehydrogenase and an apparent increase of the pK_a value (pK_0 in Scheme 1) for the ionizing group which regulates the coenzyme binding process. The significance of these two effects at sufficiently high anion concentrations is demonstrated by the sulphate inhibition data in Fig. 1. As pointed out by Dahl et al. [3], it might be of importance in some contexts to recognize the existence of such anion effects, including those attributable to the buffer solution.

Results in Fig. 4 confirm previous observations [3,4] that effects due to phosphate binding are of significant magnitude at pH 7. The present estimate of the corresponding apparent dissociation constant ($K_A = 90$ mM) agrees well with that ($K_A \approx 75$ mM) calculated from data obtained by Theorell and Winer [4] in their studies of phosphate buffer effects on the equilibrium binding of NADH. According to these estimates, coenzyme binding constants determined in 0.1 M ionic strength phosphate buffer at pH 7 would be expected to differ by a factor less than 1.6 from those determined at zero ionic strength. The apparently tighter binding of phosphate observed in the Cys-46 alkylation experiments of Dahl et al. [3] might reflect buffer effects which are not coenzyme-competitive, i.e. which do not involve complex formation at the general anion-binding site.

As concerns buffer effects at higher pH, there is one informative implication of the observation that complex formation at the general anion-binding site conforms basically to the lyotropic series of affinity order. Divalent and polyvalent anions can be anticipated to be weakly bound at the site, unless complex formation involves binding interactions additional to the charge interaction with the hydrophobic Arg-47 site. This explains why the site, despite its obvious function of interacting with the pyrophosphate group of bound coenzyme, exhibits no significant affinity for pyrophosphate buffer over the pH range (8–10) now examined. Moreover, it explains the results in Table 4 which indicate that coenzyme-competitive effects of phosphate can be attributed exclusively to the monovalent $H_2PO_4^-$ species. It may be concluded from these observations that coenzyme binding data determined in 0.1 M phosphate buffer cannot be much affected by buffer anion competition over the critical pH range (8–10) where effects of pH become pronounced. The

present results establish that the same should apply for pH dependence data reported from our laboratory [10,11,19], which have been obtained in phosphate (pH 6–9), pyrophosphate (pH 8.75), or carbonate (pH 9–10) buffer solutions of 0.1 M ionic strength. This is confirmed by and explains the results in Fig. 1 (curve A). Parameter values for NADH binding (k_1 and pK_0) now obtained from measurements in weakly buffered solution agree within experimental precision with those reported previously [10].

The present investigation, therefore, eliminates the uncertainty concerning unaccounted effects of coenzyme-competitive buffer anion binding. Such effects are not sufficiently pronounced to call for any significant reevaluation of inferences drawn from the observed pH dependence of coenzyme binding, at least not of inferences drawn from observations made in our laboratory.

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